



(51) International Patent Classification ⁶ : C12N 9/10, 15/54	A1	(11) International Publication Number: WO 9606931 (43) International Publication Date: 7 March 1996 (07.03.96)
<p>(21) International Application Number: PCT/DK95/00347</p> <p>(22) International Filing Date: 28 August 1995 (28.08.95)</p> <p>(30) Priority Data: 0990/94 26 August 1994 (26.08.94) DK 0947/95 24 August 1995 (24.08.95) DK</p> <p>(71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): BECH, Lisbeth [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). NØRREVANG, Iben, Angelica [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). HALKIER, Torben [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). RASMUSSEN, Grethe [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). SCHÄFER, Thomas [DE/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). ANDERSEN, Jens, Tønne [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK).</p>	<p>(74) Agent: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).</p> <p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</p> <p>Published <i>With international search report.</i></p>	
<p>(54) Title: MICROBIAL TRANSGLUTAMINASES, THEIR PRODUCTION AND USE</p> <p>(57) Abstract</p> <p>Transglutaminase preparations are producible by a wide range of fungi, especially ascomycotina, basidiomycotina and zygomycota, and gram-negative and gram-positive bacteria, especially <i>Streptomyces lydicus</i>, NRRL B-3446. A DNA construct encoding a novel transglutaminase and comprising the DNA sequence obtainable from the plasmid in <i>E. coli</i>, DSM 10175, is also described together with a method of producing the transglutaminases, a composition comprising the transglutaminase and a method for producing a gel or protein gelation composition.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

5 MICROBIAL TRANSGLUTAMINASES, THEIR PRODUCTION AND USE

The present invention relates to microbial transglutaminases, a DNA construct encoding a transglutaminase, a
10 method of producing the transglutaminases, a composition comprising the transglutaminase and a method for producing a gel or protein gelation composition; and the use thereof.

15 BACKGROUND OF THE INVENTION

Transglutaminases (EC 2.3.2.13) are enzymes capable of catalyzing an acyl transfer reaction in which a γ -carboxy-amide group of a peptide bound glutamine residue is
20 the acyl donor. Primary amino groups in a variety of compounds may function as acyl acceptors with the subsequent formation of monosubstituted γ -amides of peptide bound glutamic acid. When the ϵ -amino group of a lysine residue in a peptide chain serves as the acyl acceptor, the
25 transglutaminases form intramolecular or intermolecular γ -glutamyl- ϵ -lysyl crosslinks.

This peptide crosslinking activity has shown useful for a variety of industrial purposes, including gelling of proteins, improvement of baking quality of flour, producing
30 paste type food materia from protein, fat and water, preparation of cheese from milk concentrate, binding of chopped meat product, improvement of taste and texture of food proteins, casein finishing in leather processing
35 etc.

A wide array of transglutaminases have been identified and characterized from a number of animals and a few plant species. The most widely used animal derived trans-
40 glutaminase, FXIIIa, is a Ca^{2+} -dependent multi-subunit enzyme which is product inhibited, properties which are a disadvantage for many industrial applications and for

production. A Ca^{2+} -dependent transglutaminase from the slime mould *Physarum polycephalum* has been described in Klein et al., (1992).

5 Only few microbial transglutaminases have been disclosed, namely transglutaminases from the species *Streptovorticillium mobaraense*, *Streptovorticillium cinnamoneum*, and *Streptovorticillium griseocarneum* (in US 5,156,956) and from the species contemplated to be *Streptomyces lavendulae* (in US 5,252,469).

US 5,156,956 discloses that, after an extensive search for transglutaminases including screening a wide range of organisms and more than 5000 isolates of microbial origin, only the above-mentioned three *Streptovorticillium* species were found to produce transglutaminase. Members of this former genus *Streptovorticillium* are now generally included within the genus *Streptomyces* (Kaempfer et al. (1991), and Ochi et al. (1994)).

20

US 5,252,469 discloses transglutaminase from what was believed to be two related species: *Streptomyces sp.*, and *Streptomyces lavendulae*. However, from the disclosed data for the contemplated *S. lavendulae* strain it is evident to the skilled person that the disclosed strain is not *S. lavendulae*.

Streptovorticillia are classified together in Cluster group F (clusters 55 to 67) of *Streptomyces* and related genera (Williams et al.). Therefore the known microbial transglutaminases all originate from members of this Cluster group as defined in Williams et al. *Streptomyces lavendulae* is also classified in Cluster group F.

35 All known microbial transglutaminases have been identified by using a conventional enzyme assay in which hydroxylamine is converted to hydroxamic acid (Folk, J. E. & Cole, P. W. (1966)).

In order to construct strains overproducing different enzymes, recombinant DNA techniques are widely used. For the same purpose, the *Streptoverticillium mobaraense* transglutaminase gene has been cloned for expression in
5 *Escherichia coli*, *Streptomyces lividans*, and *Saccharomyces cerevisiae* (Washizu et al., Tahekana et al., and EP-A-0 481 504). Even the most succesful of these approaches (Washizu et al.) resulted in a production yield much lower than the yield in the wildtype *S. mobaraense* strain,
10 in spite of extensive experimentation and optimization. Thus, none of the efforts to overproduce the *S. mobaraense* enzyme have been successful, although they included a number of different approaches such as chemical synthesis of a codon-optimized gene and its subsequent expression
15 (as a cleavable heterologous signal peptide fusion to the mature transglutaminase) to the periplasm of *E. coli*; or expression as a similar fusion to the mature transglutaminase in *S. cerevisiae*; or expression as a similar fusion to pro-transglutaminase in *S. cerevisiae*; or
20 traditional isolation and expression of the natural DNA sequence encoding the preproenzyme in *S. lividans*.

US 5,252,469 discloses strains closely related to *S. mobaraense* which produce higher amounts of transglutaminase
25 by conventional techniques.

The object of the invention is to provide novel microbially derived transglutaminases, preferably in single-component or mono-component form, a novel gene encoding a
30 transglutaminase, and a method for producing the transglutaminase in a better yield and higher purity than hitherto possible by recombinant DNA technology, as well as the use of the transglutaminase either alone or in combination with other enzymes for the use in a variety of
35 industrial purposes, including gelling of proteins; improvement of baking quality of flour; producing paste type food or food ingredients from protein, fat and water; preparation of cheese from milk concentrate; binding

of chopped meat or fish products; improvement of taste and texture of food proteins; casein finishing in leather processing; shoe shine, etc.

5

SUMMARY OF THE INVENTION

It has been found that, by screening a wide array of bacterial and fungal strains, often screening of the same
10 extract which in the traditional hydroxamate assay gave rise to a negative result, in a modified putrescine assay resulted in a positive reaction. Accordingly, the modified version of the putrescine incorporation assay was applied in a screening procedure which surprisingly re-
15 sulted in detection of transglutaminase activity in a wide array of organisms.

Therefore, and opposite to what has hitherto been known, it has now been found that transglutaminases (TGases) are
20 produced by an overwhelming array of phylogenetically dispersed microorganisms. Also, it has been found that even within Cluster groups other than Cluster group F, e.g. Cluster groups A and G, members have been found which produce transglutaminases.

25

Several of the provided enzymes may be useful for industrial applications. The industrial potential is underlined by three circumstances:

1. The novel transglutaminases of the invention may be
30 obtained in the higher production yields than obtained for any other microbial transglutaminase;
2. A number of the TGase-producing strains provided by the mentioned assay are closely related to industrial production strains in current use, and can hence be sub-
35 jected to recombinant DNA expression in closely related species; e.g. members of the genera *Bacillus*, *Streptomyces*, *Aspergillus*, and *Trichoderma*;
3. The novel transglutaminases of the invention may be

found extracellularly.

By applying a number of different growth conditions for the organisms to be screened, the inventors also surprisingly found that these conditions, in several instances, were decisive for detection of TGase activity in the extract.

The inventors also succeeded in isolating and characterizing a DNA sequence from a strain of *Streptomyces lydicus*, exhibiting transglutaminase activity, thereby making it possible to prepare a single-component transglutaminase.

Accordingly, in another aspect the invention relates to a DNA construct comprising a DNA sequence encoding an enzyme exhibiting transglutaminase activity, which DNA sequence comprises

a) the DNA sequence shown in SEQ ID No. 1, and/or the DNA sequence obtainable from the plasmid in *E. coli* DSM 10175, or

b) an analogue of the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *E. coli* DSM 10175, which

i) is at least 80% homologous with the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *E. coli* DSM 10175, or
ii) encodes a polypeptide which is at least 79% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *E. coli* DSM 10175, or

iii) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified transglutaminase encoded by the DNA sequence shown in SEQ ID No 1 and/or obtainable from the plasmid in *E. coli*, DSM 10175.

It is believed that the DNA sequence shown in SEQ ID No. 1 is identical to the DNA sequence obtainable from the plasmid in *E. coli*, DSM 10175.

- 5 The strain *E. coli* was deposited under the deposition number DSM 10175 on 23 August 1995 at the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38125 Braunschweig, Germany, according to the Budapest Treaty.

10

In a further aspect, the invention relates to a method for the production of transglutaminase comprising cultivation in a suitable nutrient medium a strain belonging to any of the classes, orders, families, genera and species specified in the specification, examples and claims
15 herein, especially *Streptomyces lydicus*, NRRL B-3446.

The invention further relates to a transglutaminase composition comprising the transglutaminase preparation of
20 the present invention and a stabilizer.

In yet another aspect, the invention relates to a method of crosslinking proteins wherein a transglutaminase composition comprising the transglutaminase preparation of
25 the present invention is contacted with a proteinaceous substrate.

Further, the present invention relates to use of the transglutaminase preparation of the present invention in
30 flour, meat products, fish products, cosmetics, cheese, milk products, gelled food products and shoe shine.

DETAILED DESCRIPTION OF THE INVENTION

35

In the present specification and claims, the term "transglutaminase" is intended to be understood as an enzyme capable of catalyzing an acyl transfer reaction in which

a gamma-carboxyamide group of a peptide-bound glutamine residue is the acyl donor. The term "Ca²⁺-independent transglutaminase" is intended to be understood as a transglutaminase active in the absence of Ca²⁺-ions, i.e. in the presence of excess EDTA.

The transglutaminase may be a component occurring in an enzyme system produced by a given microorganism, such an enzyme system mostly comprising several different enzyme components. Alternatively, the transglutaminase may be a single component, i.e. a component essentially free of other enzyme components usually occurring in an enzyme system produced by a given microorganism, the single component being a recombinant component, i.e. produced by cloning of a DNA sequence encoding the single component and subsequent cell transformed with the DNA sequence and expressed in a host. The host is preferably a heterologous host, but the host may under certain conditions also be the homologous host.

20

The native or unmodified transglutaminase may be of microbial origin.

It is contemplated that transglutaminases may be obtainable by or derived from a fungus, a bacterium or from yeast. The derived enzyme component may be either homologous or heterologous component. Preferably, the component is homologous. However, a heterologous component which is immunologically reactive with an antibody raised against a highly purified transglutaminase and which is derived from a specific microorganism is also preferred.

In the present context the term "derivable" or "derived from" is intended not only to indicate a transglutaminase produced by a strain of the organism in question, but also a transglutaminase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Furthermore, the term is

intended to indicate a transglutaminase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the transglutaminase in question.

5

In a preferred embodiment, the invention relates to transglutaminase preparation which is producible by cultivation of a fungus, preferably a fungus which belongs to basidiomycotina, ascomycotina or zygomycotina.

10

Examples of useful basidiomycotina are strains belonging to the group consisting of the orders *Agaricales*, *Aphyllophorales*, *Ceratobasidiales*, *Auriculariales* and *Nidulariales*, or strains belonging to the group consisting of the families *Tricholomataceae*, *Amanitaceae*, *Agaricaceae*, *Strophariaceae*, *Coprinaceae*, *Cortinariaceae*, *Paxillaceae*, *Polyporaceae*, *Coriolaceae*, *Fomitopsidaceae*, *Stereaceae*, *Hymenochaetaceae*, *Lachnocladiaceae*, *Ceratobasidiaceae*, *Auriculariaceae* and *Nidulariaceae*, or strains belonging to the group consisting of the genera *Tricholoma*, *Lyophyllum*, *Armillaria*, *Amanita*, *Agaricus*, *Chamaemyces*, *Stropharia*, *Hypholoma*, *Kuhneromyces*, *Pholiota*, *Coprinus*, *Psathyrella*, *Panaeolus*, *Gymnopilus*, *Hygrophoropsis*, *Pleurotus*, *Pycnoporus*, *Antrodia*, *Trametes*, *Amylostereum*, *Hymenochaete*, *Scytinostroma*, *Rhizoctonia*, *Auricularia* and *Nidula*.

Preferred strains are those belonging to the species *Tricholoma flavovirens* or *Tricholoma myomyces*, *Lyophyllum* sp., *Armillaria* sp., *Amanita virosa*, *Agaricus* sp., *Chamaemyces fracidus*, *Stropharia coerulea*, *Hypholoma fasciculare*, *Kuhneromyces variabilis*, *Pholiota jahnii*, *Coprinus cinereus*, *Coprinus* sp., *Psathyrella condolleana*, *Panaeolus papilionaceus*, *Gymnopilus junonius*, *Hygrophoropsis aurantiaca*, *Pleurotus dryinus*, *Pleurotus* sp., *Pycnoporus cinnabarinus*, *Antrodia serialis*, *Trametes hirsuta*, *Amylostereum chailletii*, *Hymenochaete corticola*, *Scytinostroma portentosum*, *Rhizoctonia solani*, *Auricularia polytricha*

and *Nidula* sp..

Especially useful examples are those strains belonging to the group consisting of the species *Armillaria* sp., CBS 372.94; *Coprinus cinereus*, IFO 30116; *Psathyrella condoleana*, CBS 628.95; *Panaeolus papilionaceus*, CBS 630.95; *Amylostereum chailletii*, CBS 373.94; and *Hymenochaete corticola*, CBS 371.94.

- 10 Examples of useful ascomycotina are strains belonging to the classes *Discomycetes*, *Pyrenomycetes*, *Loculoascomycetes*, and *Plectomycetes*, preferably those belonging to the orders *Leotiales*, *Xylariales*, *Diaporthales*, *Sordariales*, *Halosphaeriales*, *Hypocreales*, *Dothideales*, *Eurotiales*,
15 and certain *Ascomycetes* of unknown order.

Preferred strains are strains belonging to the families *Leotiaceae*, *Xylariaceae*, *Amphisphaeriaceae*, *Valsaceae*, *Chaetomiaceae*, *Lasio-sphaeriaceae*, *Halosphaeriaceae*, *Hypocreaceae*, *Pleosporaceae*, *Mycosphaerellaceae*, *Botryosphaeriaceae*, *Sporormiaceae*, *Herpotrichiellaceae*, and *Trichocomataceae*; especially strains belonging to the genera *Dimorphosporum*, *Xylaria*, *Ascotricha*, *Nodulisporium*, *Savoryella*, *Valsa*, *Chaetomium*, *Podospora*, *Halosphaeriopsis*,
20 *Lulworthia*, *Lignincola*, *Fusarium*, *Myrothecium*, *Trichoderma*, *Alternaria*, *Cochliobolus*, *Curvularia*, *Cercospora*, *Cladosporium*, *Botryosphaeria*, *Sporormiella*, *Preussia*, *Carponia*, *Coniothyrium*, *Byssochlamys*, *Talaromyces*, *Neosartorya*, *Warcupiella*, *Aspergillus*, *Beauveria*, *Hortea*,
25 *Humicola*, *Monodictys* and *Dendryphiella*.

Preferred are the species *Dimorphosporum disparatrichum*, *Xylaria* sp., *Ascotricha erinacea*, *Nodulisporium* sp., *Savoryella lignicola*, *Valsa pini*, *Chaetomium funiculum*,
35 *Podospora tetraspora*, *Halosphaeriopsis mediosetigera*, *Lulworthia uniseptata*, *Lignincola* sp., *Fusarium armeniacum*, *Fusarium decemcellulare*, *Fusarium dimerum*, *Fusarium merismoides*, *Fusarium redolens*, *Fusarium flocciferum*,

Myrothecium roridum, *Trichoderma harzianum*, *Alternaria* alternata, *Cochliobolus sativus*, *Curvularia borreiae*, *Cercospora beticola*, *Cercospora carisis*, *Cercospora fusimaculans*, *Cercospora hayi*, *Cercospora sesami*, *Cercospora traversiana*, *Cladosporium cladosporioides*, *Cladosporium resinae*, *Cladosporium oxysporum*, *Cladosporium sphaeospermum*, *Botryosphaeria rhodina*, *Sporormiella australis*, *Sporormiella minima*, *Preussia isomera*, *Carponia solliomaris*, *Coniothyrium cerealis*, *Byssochlamys fulva*, *Talaromyces helicus*, *Neosartorya quadricineta*, *Warcuprella spinulosa*, *Aspergillus foetidus*, *Aspergillus giganteus*, *Aspergillus heteromorphus*, *Aspergillus puniceus*, *Aspergillus tamarisii*, *Beauveria cylindrospora*, *Beauveria calendonica*, *Hortea werneckii*, *Humicola alopallonella*, *Monodictys pelagica* and *Dendryphiella salina*.

Especially preferred are the species *Dimorphosporium disporatrichum*, ATCC 24562; *Savoryella lignicola*, CBS 626.95; *Chaetomium funiculum*, ATCC 42779; *Lulworthia uniseptata*, IFO 32137; *Fusarium armeniacum*, IBT 2173; *Fusarium decemcellulare*, CBS 315.73; *Fusarium dimerum*, IBT 1796; *Fusarium merismoides*, ATCC 16561; *Fusarium redolens*, IBT 2058; *Myrothecium roridum*, ATCC 20605; *Trichoderma harzianum*, CBS 223.93; *Alternaria alternata*, CBS 448.94; *Curvularia borreiae*, CBS 859.73; *Cercospora beticola*, ATCC 28056; *Cercospora carisis*, IMI 167.425; *Cercospora fusimaculans*, IMI 167.426; *Cercospora hayi*, IMI 160.414; *Cercospora sesami*, IMI 318.913; *Cercospora traversiana*, IMI 318.080; *Cladosporium resinae*, CBS 174.61; *Cladosporium sphaeospermum*, CBS 444.94; *Byssochlamys fulva*, AHU 9252; *Talaromyces helicus*, ATCC 10451; *Neosartorya quadricineta*, IBT 11057; *Warcuprella spinulosa*, NKBC 1495; *Aspergillus foetidus*, CBS 565.65; *Aspergillus giganteus*, CBS 526.65; *Aspergillus heteromorphus*, CBS 117.55; *Aspergillus puniceus*, IAM 13893; *Aspergillus tamarisii*, IBT 3824; *Beauveria cylindrospora*, CBS 719.70; *Beauveria calendonica*, CBS 485.88; *Hortea werneckii*, CBS 446.94; *Monodictys pelagica*, CBS 625.95; and Den-

dryphiella salina, CBS 447.94.

Examples of useful zygomycota are strains belonging to the order *Mucorales*, preferably strains belonging to the genera *Mucor* and *Cunninghamella*.

Preferred species are *Mucor aligarensis*, preferably ATCC 28928, *Mucor luteus* and *Cunninghamella elegans*, preferably AHU 9445.

10

As shown in the examples below, the fungal transglutaminase preparations of the invention are capable of polymerizing α -casein, also at a relatively high temperature, i.e. at temperatures where the enzyme activity is at optimum.

15

Preferred fungal transglutaminase preparations of the invention exhibit optimum activity at a temperature of at least 55°C, preferably at least 60°C, more preferably at least 70°C even more preferably at least 80°C, especially at least 90°C. Such preparations are for example producible by cultivation of strains belonging to the genera *Savoryella*, *Cladosporium*, *Monodictys*, *Hymenochaete* and *Lulworthia*, especially strains belonging to the species *Savoryella lignicola*, *Cladosporium sphaeospermum*, *Hymenochaete corticola*, *Monodictys pelagica* and *Lulworthia uni-septata*.

25

Other preferred fungal transglutaminase preparations of the invention exhibit optimum relative activity at a pH of at least 8.5, preferably at least 9.0. Such preparations are for example producible by cultivation of a strain belonging to the genera *Savoryella*, *Cladosporium*, *Cercospora*, *Hymenochaete*, *Monodictys* and *Lulworthia*.

35

Further, it is contemplated that the fungal transglutaminase activity is inhibited by phenylmethylsulfonylfluoride (PMSF).

Transglutaminases in general are thought to contain a cysteine-residue in the active site that is essential for catalysis. This is based upon the observation that compounds that react with free thiol-groups inhibit transglutaminases. These compounds are e.g. mono-iodoacetic acid or mercuri salts.

Transglutaminases inhibited by other types of compounds could have different catalytic mechanisms and thus differentiate the transglutaminases into groups analogous to the classification of the proteases. The four classes of proteases are distinguished based upon their inhibition by different compounds. For example the serine proteases are typically inhibited by phenylmethanesulfonylfluoride (PMSF) whereas the cysteine proteases are inhibited by the same compounds that inhibit transglutaminases.

In another aspect, the invention relates to a novel transglutaminase preparation which is producible by cultivation of a bacterium which, in contrast to the known microbial transglutaminases, does not belong to Cluster F of *Streptomyces* and related genera.

Preferred bacteria are gram-negative or gram-positive.

Examples of transglutaminase-producing gram-negative bacteria are strains belonging to the genera *Pseudomonas*, *Hafnia*, *Hydrogenophaga*, and *Zymomonas*.

Preferred examples of TGase-producing gram-negative bacteria are strains belonging to the species *Pseudomonas putida*, *Pseudomonas putida*, *Pseudomonas amyloclavata*, *Hafnia alvei*, *Hydrogenophaga palleroni* (Basonym: *Pseudomonas palleroni*), *Moo5A10* and *Zymomonas mobilis*; especially *Pseudomonas putida*, DSM 1693; *Pseudomonas putida*, NCIMB 9869; *Pseudomonas amyloclavata*, ATCC 21262; *Hafnia alvei*, DSM 30163; *Hydrogenophaga palleroni*, DSM 63; *Moo5A10*, DSM 10094, and *Zymomonas mobilis*, DSM 424.

Examples of TG-ase-producing gram-positive bacteria are strains belonging to the genera *Streptomyces*, *Rothia*, *Bacillus*, *Kitasatoa* and *Bacteridium*.

- 5 Preferred examples of TGase-producing gram-positive bacteria are strains belonging to the species *Streptomyces lydicus*, *Streptomyces nigrescens*, *Streptomyces sioyaensis*, *Streptomyces platensis*, *Rothia dentocariosa*, *Bacillus badius*, *Bacillus mycoides*, *Bacillus firmus*, *Bacillus*
10 *aneurinolyticus*, *Bacillus megaterium*, *Bacillus sp.*, *B. amyloliquefaciens*, *Kitasatao purpurea*, *Bacteridium sp.* and *Bacillus megaterium*.

- Most preferred are the strains *Streptomyces lydicus*, DSM
15 40555 and NRRL B-3446; *Streptomyces nigrescens*, ATCC 23941; *Streptomyces sioyaensis*, ATCC 13989; *Streptomyces platensis*, DSM 40041; *Bacillus badius*, DSM 23; *Bacillus mycoides*, GJB 371; *Bacillus firmus*, ATCC 17060; *Bacillus firmus*, DSM 12; *Bacillus aneurinolyticus*, ATCC 12856; *Ba-*
20 *cillus megaterium*, ATCC 13632; *Bacillus megaterium*, ATCC 15450; *Bacillus megaterium*, AJ 3355 and Ferm-P 1201; *Bacillus sp.*, ATCC 21537; *B. amyloliquefaciens*, ATCC 23843; *Kitasatao purpurea*, DSM 43362; *Bacteridium sp.* DSM 10093, *Bacteridium sp.*, CBS 495.74.

- 25 Preferred bacterial transglutaminase preparations of the invention exhibit optimum activity at a pH of at least 6.5, preferably at least 7.0, more preferably at least 7.5, even more preferably at least 8.0, especially at
30 least 8.5, most preferably at least 9.0.

- Further, the transglutaminase activity of preferred bacterial transglutaminase preparations of the invention is inhibited by phenylmethanesulfonylfluoride (PMSF), see
35 example 16.

Preferably, the transglutaminase is a recombinant transglutaminase, i.e. a transglutaminase essentially free

from other proteins or enzyme proteins from the parent microorganism. A recombinant transglutaminase may be cloned and expressed according to standard techniques conventional to the skilled person.

5

Advantageously, a parent transglutaminase of bacterial origin may be used, e.g. a transglutaminase derivable from a strain of the genus *Streptomyces*, *Actinoplanes*, *Amorphosporangium*, *Amycolata*, *Dactolosporangium*, *Bacteri-*
10 *dium*, *Kitasatoa*, *Micronospora*, or *Bacillus*. For instance, the parent transglutaminase may be derivable from a strain of the species *Streptomyces lydicus* (deposited at ARS Patent Culture Collection North Central Region, 1815 North University Street, Peonia, Illinois 61604, U.S.A.,
15 NRRL B-3446 (former *Streptomyces libani*).

In a preferred embodiment, the parent transglutaminase is a *Streptomyces lydicus*, NRRL B-3446, transglutaminase, or is a functional analogue of said parent transglutaminases
20 which

- i) comprises an amino acid sequence being at least 60% homologous with the amino acid sequence of the parent transglutaminase,
- 25 ii) reacts with an antibody raised against the parent transglutaminase, and/or
- iii) is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding the parent transglutaminase.

30

Property i) of the analogue is intended to indicate the degree of identity between the analogue and the parent transglutaminase indicating a derivation of the first sequence from the second. In particular, a polypeptide is
35 considered to be homologous to the parent transglutaminase if a comparison of the respective amino acid sequences reveals an identity of greater than about 60%, such as above 70%, 80%, 85%, 90% or even 95%. Sequence compari-

sons can be performed via known algorithms, such as the one described by Lipman and Pearson (1985).

The additional properties ii) and iii) of the analogue of
5 the parent transglutaminase may be determined as follows:

Property ii), i.e. the immunological cross reactivity, may be assayed using an antibody raised against or reactive with at least one epitope of the parent transglutaminase. The antibody, which may either be monoclonal or
10 polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al., 1989. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or
15 radial immunodiffusion assay, e.g. as described by Hudson et al., 1989.

The probe used in the characterization of the analogue in accordance with property iii) defined above, may suitably
20 be prepared on the basis of the full or partial nucleotide or amino acid sequence of the parent transglutaminase. The hybridization may be carried out under any suitable conditions allowing the DNA sequences to hybridize. For instance, such conditions are hybridization under specified conditions, e.g. involving presoaking in 5xSSC and
25 prehybridizing for 1h at -40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50µg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100µM ATP for 18h at -40°C, or other methods
30 described by e.g. Sambrook et al., 1989.

Other examples of parent transglutaminases are those derived from or producible by *Streptomyces platensis*, preferably
35 DSM 40041, *Streptomyces nigrescens*, preferably ATCC 23941, or *Streptomyces sioyaensis*, preferably ATCC 13989.

These parent transglutaminase are capable of polymerizing α -casein and are thus useful for many industrial purposes.

- 5 In a further aspect, the invention relates to a method for the production of transglutaminase comprising cultivation in a suitable nutrient medium a strain belonging to any of the classes, orders, families, genera and species specified herein, especially *Streptomyces lydicus*,
10 NRRL B-3446.

In yet a further aspect, the invention relates to a transglutaminase composition comprising a fungal or bacterial transglutaminase preparation as described above
15 and a stabilizer.

The invention also relates to a method of crosslinking proteins wherein a transglutaminase composition comprising the fungal or bacterial transglutaminase preparation
20 of the present invention is contacted with a proteinaceous substrate.

The transglutaminase preparation of the invention is useful in flour, meat products, fish products, cosmetics,
25 cheese, milk products, gelled food products and shoe shine.

In the present context, the "analogue" of the DNA sequence shown in SEQ ID No. 1 is intended to indicate any DNA
30 sequence encoding an enzyme exhibiting transglutaminase activity, which has any or all of the properties i)-iii). The analogous DNA sequence

a) may be isolated from another or related (e.g. the same)
35 organism producing the enzyme with transglutaminase activity on the basis of the DNA sequence shown in SEQ ID No. 1, e.g. using the procedures described herein, and thus, e.g. be an allelic or species variant of the DNA

sequence comprising the DNA sequences shown herein,

b) may be constructed on the basis of the DNA sequences shown in SEQ ID No. 1, e.g. by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the transglutaminase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. However, in the latter case amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al. (1991). Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as cysteine, glutamine and asparagine), hydrophobic amino acids (such as proline, leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

30

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or

35

alanine-scanning mutagenesis (Cunningham and Wells, (1989). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (i.e. transglutaminase) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos et al., (1992); Smith et al., (1992); Wlodaver et al., (1992).

The homology referred to in i) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970)). Using GAP e.g. with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence may exhibit a degree of identity preferably of at least 80%, more preferably at least 82%, more preferably at least 85%, especially at least 90%, with the coding region of the DNA sequence shown in SEQ ID No.1 or the DNA sequence obtainable from the plasmid in *E. coli*, DSM 10175.

The homology referred to in ii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970)). Using GAP e.g. with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1, the polypeptide encoded by an analogous DNA sequence may exhibit a degree

of identity preferably of at least 79%, more preferably at least 80%, even more preferably at least 82%, especially at least 90%, with the enzyme encoded by a DNA construct comprising the DNA sequence shown in SEQ ID No.1
5 or the DNA sequence obtainable from the plasmid in *E. coli*, DSM 10175.

In connection with property iii) above it is intended to indicate an transglutaminase encoded by a DNA sequence
10 isolated from strain DSM 10175 and produced in a host organism transformed with said DNA sequence or produced by the strain DSM 10175. The immunological reactivity may be determined by the method described in the Materials and Methods section below.

15

In further aspects the invention relates to an expression vector harbouring a DNA construct of the invention, a cell comprising the DNA construct or expression vector and a method of producing an enzyme exhibiting transglu-
20 taminase activity which method comprises culturing said cell under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

In a still further aspect the invention relates to an
25 enzyme exhibiting transglutaminase activity, which enzyme

a) is encoded by a DNA construct of the invention
b) produced by the method of the invention, and/or
30 c) is immunologically reactive with an antibody raised against a purified transglutaminase encoded by the DNA sequence shown in SEQ ID No.1 or the DNA sequence obtainable from the plasmid in *E. coli*, DSM 10175.

35 The transglutaminase mentioned in c) above may be encoded by the DNA sequence isolated from the strain *E. coli*, DSM 10175, and produced in a host organism transformed with said DNA sequence or produced by the strain *Streptomyces*

lydicus, preferably the strain *Streptomyces lydicus*, NRRL B-3446, provided by and publicly available from Agricultural Research Service Culture Collection, 1815 North University Street, Peoria, Illinois 61604, U.S.A.

5

The DNA sequence of the invention encoding an enzyme exhibiting transglutaminase activity may be isolated by a general method involving

- cloning, in suitable vectors, a DNA library
10 from *Streptomyces lydicus*,
- transforming suitable bacterial or yeast
host cells with said vectors,
- culturing the host cells under suitable con-
15 ditions to express any enzyme of interest
encoded by a clone in the DNA library,
- screening for positive clones by determining
any transglutaminase activity of the enzyme
produced by such clones, and
- isolating the enzyme encoding DNA from such
20 clones.

The general method is further disclosed in WO 94/14953
the contents of which are hereby incorporated by referen-
ce. A more detailed description of the screening method
25 is given in Example 15 below.

The DNA sequence of the DNA construct of the invention
may be isolated by well-known methods. Thus, the DNA se-
quence may, for instance, be isolated by establishing a
30 cDNA or genomic library from an organism expected to har-
bour the sequence, and screening for positive clones by
conventional procedures. Examples of such procedures are
hybridization to oligonucleotide probes synthesized on
the basis of the full amino acid sequence shown in SEQ ID
35 No. 2, or a subsequence thereof in accordance with stan-
dard techniques (cf. Sambrook et al., 1989), and/or se-
lection for clones expressing a transglutaminase activity
as defined above, and/or selection for clones producing a

protein which is reactive with an antibody raised against the transglutaminase enzyme comprising the amino acid sequence shown in SEQ ID No. 2.

- 5 A preferred method of isolating a DNA construct of the invention from a cDNA or genomic library is by use of polymerase chain reaction (PCR) using degenerate oligonucleotide probes prepared on the basis of the amino acid sequence of the parent transglutaminase enzyme. For instance, the PCR may be carried out using the techniques described in US Patent No. 4,683,202 or by R.K. Saiki et al. (1988).

Alternatively, the DNA sequence of the DNA construct of the invention may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers (1981), or the method described by Matthes et al. (1984). According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA construct may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire recombinant DNA molecule, in accordance with standard techniques.

- 30 The DNA sequence coding for the transglutaminase enzyme may for instance be isolated by screening a DNA library of *Streptomyces lydicus*, and selecting for clones expressing the appropriate enzyme activity (i.e. transglutaminase activity) or from *E. coli*, DSM 10175, deposited under the Budapest Treaty on 23 August, 1995, at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 16, D-38124 Braunschweig, Germany). The appropriate DNA sequence may then be isolated from the

clone e.g. as described in Example 1.

It is expected that a DNA sequence coding for a homologous enzyme, i.e. an analogous DNA sequence, is obtainable from other microorganisms. For instance, the DNA sequence may be derived by similarly screening a DNA library of another bacterium, preferably a gram-positive bacterium, more preferably a strain of a *Streptomyces* sp., in particular a strain of *S. platensis*.

10

Alternatively, the DNA coding for a transglutaminase of the invention may, in accordance with well-known procedures, conveniently be isolated from DNA from a suitable source, such as any of the above mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the nucleotide sequence shown in SEQ ID No. 1 or any suitable subsequence thereof.

20

The DNA sequence may subsequently be inserted into a recombinant expression vector. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

35 In the vector, the DNA sequence encoding the transglutaminase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host

- cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the transglutaminase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY, 1989).
- 10 The host cell which is transformed with the DNA sequence encoding the enzyme of the invention is preferably a eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell, or a prokaryotic cell such as a bacterial cell. In particular, the eukaryotic
- 15 cell may belong to a species of *Aspergillus*, *Fusarium* or *Trichoderma*, most preferably *Aspergillus oryzae*, *Aspergillus nidulans* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The
- 20 use of *Aspergillus* as a host microorganism is described in EP 238 023 (of Novo Nordisk A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, e.g. a strain of *Saccharomyces*,
- 25 in particular *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia* sp., *Yarrowia* sp. such as *Yarrowia lipolytica*, or *Kluyveromyces* sp. such as *Kluyveromyces lactis*. The host cell
- 30 may also be a bacterial cell, preferably a strain of gram positive bacteria, more preferably *Bacillus* or *Streptomyces*, especially *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus*
- 35 *lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans*, *S. lydicus* or *Streptomyces murinus*; or gram negative bacteria, preferably *Escherichia*,

more preferably *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

5

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are
10 well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes,
15 polyadenylation sequences operably connected to the DNA sequence encoding the protein disulfide redox agent of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

20

In a still further aspect, the present invention relates to a method of producing an enzyme according to the invention, wherein a suitable host cell transformed with a DNA sequence encoding the enzyme is cultured under conditions
25 permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host
30 cells in question. The expressed transglutaminase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the
35 medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like. The expressed transglutaminase may also be cell wall bound.

Composition of the invention

Although the useful transglutaminase may be added as such
5 it is preferred that it is formulated into a suitable
composition. The transglutaminase to be used industrially
may be in any form suited for the use in question, e.g.
in the form of a dry powder or granulate, in particular a
non-dusting granulate, a liquid, in particular a stabili-
10 zed liquid, or a protected enzyme. Granulates may be pro-
duced, e.g. as disclosed in US 4,106,991 and US 4,661,-
452, and may optionally be coated by methods known in the
art. Liquid enzyme preparations may, for instance, be
stabilized by adding nutritionally acceptable stabilizers
15 such as a sugar, a sugar alcohol or another polyol, lac-
tic acid or another organic acid according to established
methods. Protected enzymes may be prepared according to
the method disclosed in EP 238,216. The enzyme prepara-
tion of the invention may also comprise a preservative.

20

Normally, for inclusion in flour, meat products, cheese
and other milk products, fish products, cosmetics, vari-
ous gelled food, it may be advantageous that the enzyme
preparation is in the form of a dry product, e.g. a non-
25 dusting granulate, whereas for inclusion together with a
liquid it is advantageously in a liquid form.

The invention is further illustrated in the following
non-limiting examples.

30

EXAMPLE 1**Identification of microorganisms that produce transgluta-
minases**

35

The detection limit of the [1,4-¹⁴C]-putrescine incorpora-
tion assay was found to be 1/20 of the detection limit of
the hydroxamate assay.

The assay used is a slightly modified version of the original procedure (Curtis, C. G. & Lorand, L. (1976)). The transglutaminase activity is measured as incorporation of [1,4-¹⁴C]putrescine into α -casein.

5

A. Transglutaminase-producing fungi:

Transglutaminases have been identified in culture broths of several microorganisms of fungal origin using the assay described in detail below. It was not possible to detect these transglutaminase activities using the hydroxamate assay (Folk, J. E. & Cole, P. W. (1966)) as described in EP-A-0 481 504.

- 15 The fungi were inoculated into shake flasks by harvesting mycelium from PDA slants (39 g/l potato dextrose agar). The shake flasks contain either medium E (4 g/l meat extract, 4 g/l yeast extract, 40 g/l glucose, 8 g/l tryptone, 0.001 g/l FeSO₄ 7 H₂O, 2 tablets/l EBIOS, pH 7.0) ,
- 20 medium D (50 g/l potato meal, 25 g/l barley meal, 0.025 g/l BAN 800 MG, 5 g/l Na-casein, 10 g/l soy meal, 4.5 g/l Na₂HPO₄, 0.05 ml/l pluronic), medium A (75 g/l potato meal, 0.075 g/l BAN 800 MG, 40 g/l soy meal, 9 g/l Na₂HPO₄, 1.5 g/l KH₂PO₄, 0.1 ml/l pluronic), medium F (4 g/l yeast
- 25 extract, 15 g/l glucose, 1g/l K₂HPO₄, 0.5 g/l MgSO₄, pH 7,0), medium B (30 g/l soy meal, 15 g/l malto dextrine, 5 g/l bacto peptone, 0.2 g/l pluronic), medium G (Glucose 40 g/l, Soytone 10 g/l, CaCl₂ 10 mg/l, FeSO₄ 10 mg/l, MnSO₄.4H₂O 1 mg/l, ZnSO₄.7H₂O 1 mg/l, CuSO₄.5H₂O 2 mg/l,
- 30 Softwood pulp (unbleached pine) 2.5 g/l (dry weight), pH adjusted to 5.0) or medium C (KH₂PO₄ 0.2 g/l; MgSO₄, 7 H₂O 0.05 mg/l; CaCl₂, 2 H₂O 0.013 mg/l; (NH₄)H₂PO₄ 0.24 mg/l; 0.01 M Na-acetat (pH 4.5); mineral solution 7 ml/l; glucose 1 g/l; distilled water 863 ml/l; pH adjusted to 6.0;
- 35 agar 15 g/l; thiamine (after autoclaving) 1 mg/l). The cultures were cultured at 26°C for 3-30 days with shaking. The resulting culture broth were centrifuged 10 minutes at 2300 g to give cell free culture broths (trans-

glutaminase preparations).

To 20 μl of sample is added 5 μl [1,4- ^{14}C]putrescine (1.85 MBq/ml in 2% aqueous ethanol; specific activity 4.22 GBq/mmol) and 20 μl α -casein (2% in 50 mM Tris-HCl, 100 mM NaCl, 5 mM DTT, pH 7.5). Incubation takes place for 2 h at room temperature following which 30 μl of the assay mixture is spotted onto a small round Whatman 3MM filter. The filter is immediately put into a basket submerged in cold 10% trichloroacetic acid and washed for 20 min to remove excess radioactivity. After this first wash the filters are washed three times with cold 5% trichloroacetic acid, one time with cold ethanol:acetone (50:50, v:v) and one time with cold acetone. Each of these washes takes place for 5 min. In all washing steps the amount of washing liquid should be at least 5 ml/filter. The washed filters are counted directly in scintillation vials.

Units: An enzyme activity which incorporates 1 nmol [1,4- ^{14}C]-putrescine per hour is defined as 1 U.

The tables below disclose species that produce transglutaminases in the specified growth medium upon cultivation. The enzyme activities are shown in terms of units of transglutaminase activity.

Transglutaminase positive basidiomycotina:

Class: Hymenomycetes
Order: Agaricales

	Family: Agaricaceae	Medium	Units/ml	Dep.No.
	Agaricus sp.	A	0.15	-
35	Chamaemyces fracidus	B	0.20	-

Family: Amanitaceae

	Amanita virosa	B	0.14	-
--	----------------	---	------	---

Family: Coprinaceae

	Coprinus cinereus	B	0.14	IFO 30116
	Coprinus sp.	C	0.44	-
	Psathyrella	B	0.19	CBS 628.95
5	condolleana			
	Panaeolus	C	0.15	CBS 630.95
	papilionaceus			

Family: Strophariaceae

10	Stropharia coerulea	B	0.16	-
	Hypholoma fasciculare	B	0.14	-
	Kuhneromyces	B	0.14	-
	variabilis			
	Pholiota jahnii	B	0.21	-

15

Family: Tricholomataceae

	Tricholoma			
	flavovirens	B	0.16	-
	Tricholoma myomyces	B	0.23	-
20	Lyophyllum sp.	D	0.25	-
	Armillaria sp.	D	0.62	CBS 372.94

Family: Polyporaceae

	Pleurotus dryinus	B	0.22	-
25	Pleurotus sp.	B	2.36	-

Family: Paxillaceae

	Hygrophoropsis			
	aurantiaca	B	0.17	-
30				

Family: Cortinariaceae

	Gymnopilus junonius	B	0.20	-
--	---------------------	---	------	---

Order: Aphyllophorales

35

Family: Coriolaceae

	Pycnoporus			
	cinnabarinus	B	0.14	

Family: Fomitopsidaceae

Antrodia serialis	D	0.80	
Trametes hirsuta	B	0.21	

5 Family: Stereaceae

Amylostereum chailletii	D	0.44	CBS 373.94
----------------------------	---	------	------------

Family: Hymenochaetaceae

10 Hymenochaete corticola	C	1.31	CBS 371.94
------------------------------	---	------	------------

Family: Lachnocladiaceae

15 Scytinostroma portentosum	B	0.14	-
---------------------------------	---	------	---

Order: Ceratobasidiales**Family: Ceratobasidiaceae**

20 Rhizoctonia solani	D	0.17	-
-----------------------	---	------	---

Order: Auriculariales**Family: Auriculariaceae**

25 Auricularia polytricha	A	0.18	-
------------------------------	---	------	---

Class: Gasteromycetes**Order: Nidulariales**

30

Family: Nidulariaceae

Nidula sp.	B	0.14	-
------------	---	------	---

35 Transglutaminase positive ascomycetes:

Class: Loculoascomycetes**Order: Dothideales****Family: Pleosporaceae**

5	<i>Alternaria alternata</i>	A	0.16	CBS 448.94
	<i>Cochliobolus sativus</i>	B	0.09	-
	<i>Curvularia borreiae</i>	D	0.28	CBS 859.73

Family: Mycosphaerellaceae

10	<i>Cercospora beticola</i>	A	1.58	ATCC 28056
	<i>Cercospora carisis</i>	A	13.0	IMI 167.425
	<i>C. fusimaculans</i>	A	1.3	IMI 167.426
	<i>Cercospora hayi</i>	A	0.26	IMI 160.414
	<i>Cercospora sesami</i>	A	0.24	IMI 318.913
15	<i>C. traversiana</i>	A	0.53	IMI 318.080
	<i>Cladosporium</i>			
	<i>cladosporiodes</i>	A	0.22	-
	<i>Cladosporium resinae</i>	B	0.14	CBS 174.61
	<i>C. oxysporum</i>	A	0.19	-
20	<i>C. sphaeospermum</i>	A	1.07	CBS 444.94

Family: Botryosphaeriaceae

	<i>Botryosphaeria</i>			
	<i>rhodina</i>	A	0.32	-
25				

Family: Sporormiaceae

	<i>Sporormiella</i>			
	<i>australis</i>	E	0.13	-
	<i>Sporormiella minima</i>	D	0.20	-
30	<i>Preussia isomera</i>	D	0.22	-

Family: Herpotrichiellaceae

	<i>Carponia solliomaris</i>	A	0.11	-
--	-----------------------------	---	------	---

Family: Unknown family

35	<i>Coniothyrium cerealis</i>	D	0.13	-
----	------------------------------	---	------	---

Class: Pyrenomycetes**Order: Xylariales***Family: Xylariaceae*

5	Xylaria sp	E	0.28	-
	Ascotricha erinacea	A	0.15	-
	Nodulisporium sp.	D	1.20	-

Family: Amphisphaeriaceae

10	Savoryella lignicola	A	6.24	CBS 626.95
----	----------------------	---	------	------------

Order: Diaporthales*Family: Valsaceae*

15	Valsa pini	D	0.73	-
----	------------	---	------	---

Order: Sordariales*Family: Chaetomiaceae*

20	Chaetomium funiculum	B	0.16	ATCC 42779
----	----------------------	---	------	------------

Family: Lasiosphaeriaceae

	Podospora tetraspora	D	0.30	-
--	----------------------	---	------	---

25 Order: Halosphaeriales*Family: Halosphaeriaceae*

	Halosphaeriopsis			
	mediosetigera	D	0.34	-
30	Lulworthia			
	uniseptata	E	0.36	IFO 32137
	Lignincola sp.	D	0.15	-

Order: Hypocreales

35

Family: Hypocreaceae

	Fusarium armeniacum	D	0.19	IBT 2173
	Fus. decemcellulare	B	0.10	CBS 315.73

32

	<i>Fusarium dimerum</i>	B	0.25	IBT 1796
	<i>Fusarium merismoides</i>	D	0.16	ATCC 16561
	<i>Fusarium redolens</i>	B	0.16	IBT 2059
	<i>Fusarium flocciferum</i>	D	0.15	-
5	<i>Myrothecium roridum</i>	B	0.13	ATCC 20605
	<i>Trichoderma harzianum</i>	A	0.25	CBS 223.93

Class: Discomycetes10 **Order: Leotiales***Family: Leotiaceae**Dimorphosporum*

15	<i>disporatrichum</i>	A	0.42	ATCC 24562
----	-----------------------	---	------	------------

Class: Plectomycetes**Order: Eurotiales***Family: Trichocomataceae*

20	<i>Byssoschlamys fulva</i>	A	0.24	AHU 9252
	<i>Talaromyces helicus</i>	B	0.23	ATCC 10451
	<i>Neosartorya</i>			
	<i>quadricineta</i>	D	0.22	IBT 11057
	<i>Warcupiella spinulosa</i>	D	0.41	NKBC 1495
25	<i>Aspergillus foetidus</i>	A	0.23	CBS 565.65
	<i>Aspergillus giganteus</i>	C	0.19	CBS 526.65
	<i>Asp. heteromorphus</i>	C	0.12	CBS 117.55
	<i>Aspergillus puniceus</i>	F	0.12	IAM 13893
	<i>Aspergillus tamarai</i>	A	0.16	IBT 3824

30

Order: Ascomycetes of unknown order*Beauveria*

	<i>cylindrospora</i>	A	0.24	CBS 719.70
35	<i>Beauveria calendonica</i>	A	0.25	CBS 485.88
	<i>Hortea werneckii</i>	A	0.95	CBS 446.94
	<i>Humicola</i>			
	<i>alopallonella</i>	C	0.76	-

Monodictys pelagica	A	2.31	CBS 625.95
Dendryphiella salina	D	0.96	CBS 447.94

Transglutaminase positive zygomycetes:

5

Order: Mucorales

Mucor aligarensis	D	0.31	ATCC 28928
Mucor luteus	B	0.34	-
Cunninghamella			
elegans	B	0.23	AHU 9445

B. Transglutaminase-producing bacteria:

- 15 Bacteria grown on Tryptone-yeast agar-plates were used for inoculation of shake flasks. The shake flasks contained 100 ml the media listed below. Cultures were incubated at 30°C for 1-12 days while shaking at 250 rpm. Samples (5 ml) were taken from the broth and analyzed for
- 20 Tgase activity either in the crude broth, in cell-free supernatant (after centrifugation for 15 min at 2300 x g) or in the cell-pellet which was resuspended in an equal amount of sterile medium.
- 25 The table below shows examples of bacterial species that produce TGase upon cultivation in the listed media. Tgase activity is given in units/ml.

Genus/species	medium	units/ml	Dep.No.
30 <i>Streptomyces lydicus</i>	H	1.3	DSM 40555 NRRL B-3446
<i>Streptomyces nigrescens</i>	A	0.3	ATCC 23941
<i>Streptomyces sioyaensis</i>	H	3.3	ATCC 13989
35 <i>Streptomyces platensis</i>	A	1.4	DSM 40041
<i>Rothia dentocariosa</i>	J	0.9	-
<i>Bacillus badius</i>	K	0.8	DSM 23
<i>Bacillus mycoides</i>	L	0.4	GJB 371

34

	<i>Bacillus firmus</i>	J	0.6	ATCC 17060
	<i>Bacillus firmus</i>	J	0.03	DSM 12
	<i>Bacillus aneurinolyticus</i>	N	0.8	ATCC 12856
	<i>Bacillus megaterium</i>	J	0.02	ATCC 13632
5	<i>Bacillus megaterium</i>	J	0.02	ATCC 15450
	<i>Bacillus megaterium</i>	J	0.03	AJ 3355
				Ferm-P 1201
	<i>Bacillus sp.</i>	J	0.1	ATCC 21537
	<i>B. amyloliquefaciens</i>	P	0.06	ATCC 23843
10	<i>Kitasatao purpurea</i>	P	0.3	DSM 43362
	<i>Bacteridium sp. (1)</i>	A	0.3	DSM 10093
	<i>Bacteridium sp. (1)</i>	Q	0.5	CBS 495.74
	<i>Pseudomonas putida</i>	A	0.84	DSM 1693
	<i>Pseudomonas putida</i>	D	1.4	NCIMB 9869
15	<i>Pseudomonas amyloclavata</i>	N	0.08	ATCC 21262
	<i>Hafnia alvei</i>	K	0.3	DSM 30163
	<i>Hydrogenophaga palleroni</i>	Q	0.6	DSM 63
	(Basonym: <i>Pseudomonas palleroni</i>)			
	<i>Zymomonas mobilis</i>	N	0.36	DSM 424
20	<i>Moo5A10 (1)</i>	L	0.44	DSM 10094

Note (1): These strains are most probable *Bacillus* strains.

25 The used media were:

Medium	Compound	Amount	pH
N	Tryptone	20 g	7.0
	soluble starch	20 g	
30	KH ₂ PO ₄	1 g	
	MgSO ₄	1 g	
	Yeast extract	2 g	
	Pluronic 100%	0.5 g	
	Aqua dest	1000 ml	
35			
J	Trypticase	40 g	7.3
	yeast extract	10 g	
	FeCl ₂ x 4H ₂ O, 1% sol.	1.2 ml	

35

		MnCl ₂ x 4H ₂ O, 1% sol.	0.2 ml	
		MgSO ₄ x 7H ₂ O, 1% sol.	3 ml	
		Aqua dest.	1000 ml	
5	K	Casitone	3 g	6.5
		CaCl ₂ x 2 H ₂ O	0.5 g	
		MgSO ₄ x 7 H ₂ O	2 g	
		Cyanocobalamine	2 g	
		Trace elements (1)	1 ml	
10		Aqua dest	1000 ml	
	Q	Casitone	1 g	6.5
		Yeast extract	0.5 g	
		CaCl ₂ x 2 H ₂ O	0.5 g	
15		MgSO ₄ x 7 H ₂ O	0.5 g	
		Glucose	2 g	
		Aqua dest	1000 ml	
	L	Soluble starch	15g	7.0
20		NaCl	5 g	
		Corn steep liquid	10 g	
		Crushed soy bean	10g	
		CaCO ₃	2 g	
		Pluronic 100 % sol.	0.1 ml	
25		Tap water	1000 ml	
	H	Glucose	10 g	7.0
		Soluble starch	30 g	
		Yeast extract	7 g	
30		Polypeptone	7 g	
		NaCl	3 g	
		CaCO ₃	5 g	
		Tap water	1000 ml	
35	P	Peptone	6 g	7.3
		Peptidase	4 g	
		Yeast extract	3 g	
		Beef extract	1.5 g	

Dextrose	1 g
Aqua demineralised	1000 ml

Trace element solution for Medium K (mg/l Milli Q water):

5 $\text{MnCl}_2 \times 4 \text{ H}_2\text{O}$: 100, $\text{CoCl} \times 6 \text{ H}_2\text{O}$: 36.34, $\text{CuSO}_4 \times 5 \text{ H}_2\text{O}$:
15.64, $\text{Na}_2\text{MoO}_4 \times 2 \text{ H}_2\text{O}$: 10, ZnCl_2 : 20, LiCl : 5, $\text{SnCl}_2 \times 2$
10 H_2O : 5, H_3BO_3 :310, KBr : 20, KI : 20, $\text{Na}_2\text{-EDTA}$: 8.

10 EXAMPLE 2

Media-dependent expression of transglutaminases

The amount of transglutaminase activity found in culture
broths of the microorganisms was found to depend on the
15 growth media used for cultivation. This is believed to be
valid for all microorganisms, i.e. fungi or bacteria.

A. Fungi:

The table below shows the transglutaminase activity found
20 in the culture broth of the fungi *Hymenochaete corticola*
and *Cercospora carisis*, respectively, cultivated on three
different media (see example 1 for the compositions of
the used media).

25	Strain	Medium	Activity (U/ml)
	<i>Hymenochaete cor-</i> <i>ticola</i>	C	1.31
	<i>Hymenochaete cor-</i> <i>ticola</i>	B	0
30	<i>Hymenochaete cor-</i> <i>ticola</i>	G	0
	<i>Cercospora carisis</i>	C	0.3
	<i>Cercospora carisis</i>	B	0.4
	<i>Cercospora carisis</i>	A	13

B. Bacteria:

Also for bacteria the amount of TGase activity found in the culture broths of the bacteria was found to depend on the growth media used for cultivation.

5

Selected strains were grown in the different media (see above example 1) to investigate the effect of the medium on the expression of TGase activity. In the following table an example is given for *Pseudomonas putida* and *Hydrogenophaga palleroni* TGase. The other strains investigated were *Streptomyces lydicus*, *Pseudomonas putida* (DSM 1693), *Rothia dentocariosa*, *Bacillus firmus*, *Bacillus badius*, *Bacillus amyloliquefaciens*, *Bacillus aneurinolyticus*, *Bacillus megaterium* (3 strains, see example 1), *Bacillus mycoides*, *Zymomonas mobilis*, *Hafnia alvei*, *Kitasatoa purpurea*, *Bacteridium* sp., strain Moo5A10.

	Medium	Activity (U/ml)	Activity (U/ml)
		<i>Ps. putida</i> (NCIMB 9869)	<i>Hy. palleroni</i>
20	N	0	0
	J	0.08	0
	A	1.4	0
	D	1.4	0
	Q	1.8	0.6
25	L	0.12	0.18
	H	0.4	0
	P	0.2	0.07

30 **EXAMPLE 3****A. Temperature dependency of fungal transglutaminases**

The temperature dependency of the transglutaminase present in the transglutaminase preparation of *Cladosporium sphaeospermum*, *Cercospora carisis*, *Savoryella lignicola* and *Lulworthia unisepta* (see example 1 for deposition numbers) was investigated using a modification of the

35

putrescine assay described in example 1.

For determination of the temperature dependency incubation took place for 1 hour at either room temperature,
 5 40°C, 55°C, 60°C, 70°C, 80°C or 90°C.

The table below shows the temperature dependencies of the fungal transglutaminases. The enzyme activities are given in relative activities.

10

Strain	Relative activities at temperature						
	RT	40° C	55° C	60° C	70° C	80° C	90° C
15 <i>Cercospora carisis</i>	33	100	54	21	20	27	8
<i>Cladosporium sphaeospermum</i>	19	41	58	68	84	100	41
<i>Savoryella lignicola</i>	17	75	100	88	12	10	6
20 <i>Lulworthia uniseptata</i>	7	15	24	27	41	69	100
<i>Hymenochaete corticola</i>	8	17	29	40	46	52	100
25 <i>Monodictys pelagica</i>	23	77	100	47	36	73	74

B. Temperature dependency of bacterial transglutaminases

30 In this experiment temperature-dependencies of Tgases were examined in the following strains: *Pseudomonas putida* (NCIMB 9869), *Bacteridium* sp. (DSM 10093), strain

Moo5A10, *Bacillus firmus*, *Bacillus badius* and *Rothia dentocariosa*.

5 TGase containing samples from these strains were assayed at 20, 30, 40 and 55°C (2 h of incubation). Samples were either cell-free culture fluid (*Pseudomonas putida*, *Bacteridium* sp., strain Moo5A10), centrifuged cells resuspended in sterile medium (*Bacillus firmus*, *Bacillus badius*) or crude culture broth (*Rothia dentocariosa*).

10

Strain	20°C	30°C	40°C	55°C
<i>Pseudomonas putida</i>	31	100	55	55
<i>Bacteridium</i> sp.	53	77	100	78
Moo5 A10	83	85	100	96
15 <i>Bacillus firmus</i>	70	100	68	41
<i>Bacillus badius</i>	46	48	100	33
<i>Rot. dentocariosa</i>	78	67	70	100

20 EXAMPLE 4

A. pH dependency of fungal transglutaminases

The pH dependency of the transglutaminase present in the transglutaminase preparation of *Cladosporium sphaeospermum*, *Cercospora carisis*, *Savoryella lignicola* and *Lulworthia unisepta* was investigated using a modification of the putrescine assay described in example 1.

30 A 4% α -casein solution was made in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5 and diluted 1:1 in a modified 200 mM Britton-Robinson buffer (0.1M CH₃COOH, 0.2 M H₃BO₃) at the pH values mentioned below. Before assaying, CaCl₂ and cysteine were added to a final concentration of 5 mM and 1
35 mM, respectively.

For pH dependency determination incubation takes place at room temperature for 1 hour at pH 7.0, 7.5, 8.0, 8.5 or

9.0.

The table below shows the pH dependencies of the fungal transglutaminases. The enzyme activities are given in relative activities.

Strain	Relative activities at pH				
	7.0	7.5	8.0	8.5	9.0
<i>Cercospora carisis</i>	77	78	78	100	66
<i>Cladosporium sphaeospermum</i>	53	56	67	n.d	100
<i>Savoryella lignicola</i>	63	69	75	n.d	100
<i>Lulworthia uniseptata</i>	15	21	39	n.d.	100
<i>Hymenochaete corticola</i>	11	12	24	n.d.	100
<i>Monodictys pelagica</i>	15	32	43	n.d.	100

n.d.: not determined.

B. pH dependency of bacterial transglutaminases

TGase activities from selected strains were investigated using modified Britton-Robinson buffer adjusted to pH 6.5, 7, 7.5, 8, 8.5 and 9. A 4% α -casein solution was made in 50 mM Tris/HCl, 100 mM NaCl, pH 7.5 and diluted 1:1 in 200 mM Britton-Robinson buffer (0.1 M CH₃COOH, 0.2 M H₃BO₃) at the pH values mentioned above.

The TGase activity was measured at 20°C in a standard assay with 2 hours incubation and 2 mM EDTA. The strains investigated were *Bacteridium* sp., Moo5A10, *Bacillus firmus*, *Bacillus mycoides*, *Bacillus badius*, *Bacillus aneuris-*

nolyticus, *Rothia dentocariosa*.

Results:

5	Strain	Relative activity (%)					
		pH6.5	pH7	pH7.5	pH8	pH8.5	pH 9
	<i>Bacteridium sp.</i>	72	85	76	86	88	100
	<i>Moo5A10</i>	37	38	44	59	76	100
	<i>Bacillus firmus</i>	15	20	28	40	70	100
10	<i>Bacillus mycoides</i>	63	78	66	84	100	67
	<i>Bacillus badius</i>	43	46	49	65	76	100
	<i>B. aneurinoliticus</i>	47	47	57	39	100	72
	<i>Rot. dentocariosa</i>	100	63	62	64	61	38

15

EXAMPLE 5

A. Ca^{2+} -dependency of fungal transglutaminases.

- 20 The Ca^{2+} -dependency of the transglutaminase present in the transglutaminase preparation of *Cercospora carisis* and *Savoryella lignicola* was investigated using the modified putrescine assay described in example 1.
- 25 The transglutaminase preparations were concentrated approximately 10 times using a Macrosep™ concentrators from Filtron. Following the samples were diluted 10 times in 50 mM Tris-HCl, 100 mM NaCl, 1 mM cysteine, pH 7.5 ($-\text{Ca}^{2+}$) or in 50 mM Tris-HCl, 100 mM NaCl, 1 mM cysteine +
- 30 5 mM CaCl_2 , pH 7.5 ($+\text{Ca}^{2+}$).

For determination of the Ca^{2+} -dependency the incubation took place at 40°C for 1 hour. The α -casein was dissolved in either 50 mM Tris-HCl, 100 mM NaCl, 1 mM cysteine, pH

35 7.5 or in 50 mM Tris-HCl, 100 mM NaCl, 1 mM cysteine + 5 mM CaCl_2 , pH 7.5

The results are shown in the table below:

Strain	Relative activities	
	- Ca ²⁺	+ Ca ²⁺
<i>Savoryella lignicola</i>	70	100
<i>Cercospora carisis</i>	99	100

5

B. Ca²⁺ dependency of bacterial transglutaminases

The effect of Ca²⁺-ions on Tgase activity was investigated in selected Ca²⁺-free samples derived after centricon treatment. The samples were applied to a 10 kD Centricon concentrator, centrifuged (no activity in the filtrate), the enzymes in the retentate were resuspended in an equal amount of Ca²⁺-free Tris-buffer (0.1 M, pH 7.5) and centrifuged again to resolve them from the filtrate. All samples were concentrated and diluted for a second time in order to ensure Ca²⁺-free conditions.

Samples from the second centricon treatment were incubated both in the presence (2 mM CaCl₂) and in the absence of Ca²⁺ (5 mM EDTA) to determine Ca²⁺-effects. TGase activity was measured before the centricon treatment (set to 100%) and after first and second centricon treatment. Only selected strains were investigated: *Bacteridium*, *Moo5A10*, *Bacillus firmus* and *Bacillus badius*.

The results were:

species	Relative TGase activity					
	bef. centr.	after 1st centricon		after 2nd centricon		
		-Ca++	+Ca++	-Ca++	+Ca++	
<i>Bacteridium sp.</i>	100	99	n.d.	76	129	

30

43

<i>Moo5A10</i>	100	96	n.d.	104	112
<i>Bacillus firmus</i>	100	78	n.d.	60	79
<i>Bacillus badius</i>	100	5	52	n.d.	n.d.

n.d. = not determined

5

In this experiment TGases investigated from *Bacteridium*, *Moo5A10* and *Bacillus firmus* are Ca^{2+} -independent: Activity after filtration in an EDTA containing assay was about as high as before centrifugation in the untreated sample (80-99 %). After 2nd centricon the activity is slightly stimulated by the addition of Ca^{2+} for *Moo5A10* and *Bacteridium*. For *Bacteridium* still about 80 % and for *Moo5A10* about 93% of activity were measured without Ca^{2+} . Therefore these activities are defined as Ca^{2+} -independent.

Bacillus badius TGase was Ca^{2+} -dependent: after first centricon no activity (5%) was measured without added Ca^{2+} . The activity could be restored after adding 2 mM Ca^{2+} to about 50 %.

EXAMPLE 6

25 Polymerization of casein with transglutaminases

The cell free culture broths of several selected transglutaminase producing microorganisms were investigated for their ability to polymerize casein in solution. In addition, two purified microbial transglutaminases were also investigated.

In general, 100 μl sample were mixed with 20 μl 0.1M glutathion in 0.2 M Tris-HCl, pH 7.9 and 100 μl 1.5% α -casein in 0.2 M Tris-HCl, pH 7.9 and incubated for various times at 37 °C. The reaction was stopped by mixing 20 μl incubation mixture with 20 μl sample buffer for SDS-PAGE analysis followed by heating at 95 °C for 10 min. The

polymerization was visualised by SDS-PAGE.

The fermentation broths investigated were from *Streptomyces lydicus*, *Cercospora carisis*, *Cladosporium sphaeospermum* and *Savoryella lignicola* while the purified samples
5 were from *Streptomyces lydicus* and *Streptomyces platensis*.

The experiment was also carried out with fermentation
10 broth from *Lulworthia uniseptata* with the only difference being that the incubation took place at 70 °C and at 90°C. In addition, the experiment was carried out with fermentation broth from *Cladosporium sphaeospermum* with incubation at 80°C.

15

In all cases the incubation resulted in the rapid formation of casein polymers of very high molecular mass formed concomitant with the reduction of α -casein monomers.

20

EXAMPLE 7

Purification of the transglutaminase from *Streptomyces lydicus*, NRRL B-3446 (former *Streptomyces libani*)

25

Streptomyces lydicus, NRRL B-3446 (former *Streptomyces libani*), was inoculated into 1 l Zym medium (20 g/l yeast extract, 12 g/l glucose, 10 g/l bactopectone, 0.01% pluronic, pH 6.5) and cultured with shaking at 30 °C for 24
30 h. The resulting seed culture solution was added to 16 l of Zym medium which was then cultured with shaking at 30 °C for 4 days. The resulting culture broth was filtered to give 11.8 l of culture filtrate. The transglutaminase activity in the culture filtrate was 3 U/ml.

35

The culture filtrate was concentrated six times using a Filtron Minisette membrane with 3 kDa cut off. From a 500 ml portion of the concentrate the transglutaminase was

precipitated by adding ammonium sulfate to 65% saturation at ambient temperature. The precipitate was dissolved in 10 mM sodium acetate pH 6.0. After extensive dialysis against 10 mM sodium acetate pH 6.0 the sample was passed
5 through a SP-Sepharose column equilibrated with 10 mM sodium acetate pH 6.0. The transglutaminase was eluted using a linear gradient from 0 to 0.5 M sodium chloride. Fractions with high specific activity were collected and the pool was concentrated in an Amicon cell equipped with
10 a Diaflo membrane with 10 kDa cut off. A buffer change to 20 mM sodium phosphate pH 6.5 was made in the Amicon cell. The last impurities in the preparation was removed by passing it through a Blue-Sepharose column equilibrated with 20 mM sodium phosphate pH 6.5. The transglutami-
15 nase was eluted using a linear gradient from 0 to 1.0 M sodium chloride. The enzyme was pure as judged by SDS-PAGE and N-terminal sequencing. The specific activity of the pure transglutaminase was 90 times that of the culture filtrate.

20

EXAMPLE 8

25 Purification of the Ca^{2+} -independent transglutaminase from *Streptomyces platensis*

Streptomyces platensis was inoculated into 500 ml H medium (7 g/l yeast extract, 10 g/l glucose, 7 g/l polypeptone, 30 g/l soluble starch, 3 g/l NaCl, 5 g/l CaCO_3 , pH
30 7.0) and cultured with shaking at 30 °C for 24 h. The resulting seed culture solution was added to 8 l of H medium which was then cultured with shaking at 30 °C for 2 days. The resulting culture broth was filtered to give 5.0 l of culture filtrate. The transglutaminase activity
35 in the culture filtrate was 2.4 U/ml.

The culture filtrate was concentrated to 300 ml using a Filtron Minisette membrane with 3 kDa cut off. After ex-

tensive dialysis against 10 mM sodium acetate, pH 5.5 the sample was passed through an S-Sepharose column equilibrated with 10 mM sodium acetate, pH 5.5. The transglutaminase was eluted using a linear gradient from 0 to 0.25 M sodium chloride. Fractions with high specific activity were collected and the pool was dialysed against 10 mM Tris-HCl, pH 9.0. The pool was applied in 5 ml aliquots to a 1 ml Mono-Q Sepharose column equilibrated with 10 mM Tris-HCl, pH 9.0. The transglutaminase was eluted using a linear gradient from 0 to 0.25 M sodium chloride. The transglutaminase containing fractions were pooled and concentrated in an Amicon cell equipped with a Diaflo membrane with a 10 kDa cut off. A buffer change to 100 mM sodium phosphate, pH 6.5 was made in the Amicon cell. The last impurities in the preparation was removed by gel filtration using a Superdex 75 column equilibrated in 100 mM sodium phosphate, pH 6.5. The enzyme was pure as judged by SDS-PAGE and N-terminal sequencing. The specific activity relative to the culture filtrate was 800 times that of the culture broth.

The temperature optimum was found to be 45 °C and the pH optimum was found to be above pH 9.

25

EXAMPLE 9

Inhibition of the Ca^{2+} -independent transglutaminase from *Streptomyces platensis*

30

As transglutaminases in general are considered to be dependent on the presence of a free Cys-residue the transglutaminase from *Streptomyces platensis* was incubated in the presence and absence of nine fold molar excess of inhibitor (50 μM) to cysteine. Four cysteine reactive compounds were used mono-iodoacetic acid, ZnCl_2 , HgCl_2 , and FeCl_3 . Samples were incubated in the putrescine assay with and without inhibitor for 2 h at room temperature

before the activity was measured. The incubations were carried out in duplicate.

In samples incubated with mono-iodoacetic acid, ZnCl_2 , or
5 HgCl_2 , no residual activity was found. In the samples with
 FeCl_3 , less than one percent residual activity was found.
This is different from the results obtained by Ando et
al. (Agric. Biol. Chem. 53(10), 2313-2317, 1989) with the
transglutaminase from *S. mobaraense*. These authors find
10 76%, 89% and 11% residual activity after preincubation of
the transglutaminase for 30 min at 25 °C with 1 mM mono-
iodoacetic acid, 1 mM FeCl_3 , and 1 mM ZnCl_2 , respectively.
Thus, the inhibition profiles of the two transglutamina-
ses are clearly different.

15

EXAMPLE 10

Structural characterization of the transglutaminase from *Streptomyces lydicus*

20

Structural characterization of the transglutaminase was
carried out on a small amount of highly purified enzyme
(1.5 ml; $A_{280} = 0.3$). One fifth was used for direct N-ter-
minal amino acid sequencing. The remaining material was
25 lyophilized and redissolved in 350 μl 6M guanidinium
chloride, 0.3 M Tris-HCl, pH 8.3 and denatured overnight
at 37 °C. The solution was added 10 μl 0.1 M DTT and in-
cubated for 4 h at room temperature before addition of 20
 μl 0.5 M freshly prepared ICH_2COOH . The reduced and S-
30 carboxymethylated sample was desalted using a NAP5 column
(Pharmacia) equilibrated and eluted with 20 mM NH_4HCO_3 .

Following vacuum concentration the S-carboxymethylated
transglutaminase was degraded for 16 h at 37 °C with 10
35 μg of lysine-specific protease (*Achromobacter* protease
I). The resulting peptides were fractionated using rever-
sed phase HPLC on a Vydac C18 column eluted with a linear
gradient of 80% 2-propanol in 0.1% TFA. Selected peptide

fractions were subjected to repurification using reversed phase HPLC on another Vydac C18 column eluted with linear gradients of 80% acetonitrile in 0.1% TFA.

- 5 N-terminal amino acid sequencing of the intact transglutaminase as well as sequencing of the purified peptides were done in an Applied Biosystems 473A protein sequencer operated according to the manufacturers instructions.
- 10 The sequences obtained are the following:

N-terminal sequence:

Ala-Ala-Asp-Glu-Arg-Val-Thr-Pro-Pro-Ala-Glu-Pro-Leu-Asn-
Arg-Met-Pro-Asp-Ala-Tyr-Arg-Ala-Tyr-Gly-Gly-Arg-Ala-Thr-
15 Thr-Val-Val-Asn-Asn-Tyr-Ile-Arg-Lys-Trp-Gln-

Peptide 1:

Trp-Gln-Gln-Val-Tyr-Ala-His-Arg-Asp-Gly-Ile-Gln-Gln-Gln-
Met-Thr-Glu-Glu-Gln-Arg-Glu-
20

Peptide 2:

Leu-Ala-Phe-Ala-Phe-Phe-Asp-Glu-Asn-Lys

25 *Peptide 3:*

Ser-Asp-Leu-Glu-Asn-Ser-Arg-Pro-Arg-Pro-Asn-Glu-Thr-Gln-
Ala-Glu-Phe-Glu-Gly-Arg-Ile-Val-Lys

Peptide 4:

30 Gly-Phe-Lys

Peptide 5:

Ala-Leu-Asp-Ser-Ala-His-Asp-Glu-Gly-Thr-Tyr-Ile-Asp-Asn-
Leu-Lys
35

Peptide 6:

Thr-Glu-Leu-Ala-Asn-Lys

Peptide 7:

Asn-Asp-Ala-Leu-Arg-Tyr-Glu-Asp-Gly-Arg-Ser-Asn-Phe-Tyr-
Ser-Ala-Leu-Arg-Asn-Thr-Pro-Ser-Phe-Lys

5 *Peptide 8:*

Glu-Arg-Asp-Gly-Gly-Asn-Tyr-Asp-Pro-Ser-Lys

Peptide 9:

Ala-Val-Val-Tyr-Ser-Lys

10

Peptide 10:

His-Phe-Trp-Ser-Gly-Gln-Asp-Gln-Arg-Gly-Ser-Ser-Asp-Lys

Peptide 11:

15 Tyr-Gly-Asp-Pro-Asp-Ala-Phe-Arg-Pro-Asp-Gln-Gly-Thr-Gly-
Leu-Val-Asp-Met-Ser-Lys

Peptide 12:

Asp-Arg-Asn-Ile-Pro-Arg-Ser-Pro-Ala-Gln-Pro-Gly-Glu-Ser-
20 Trp-Val-Asn-Phe-Asp-Tyr-Gly-Trp-Phe-Gly-Ala-Gln-

Peptide 13:

Thr-Ile-Trp-Thr-His-Ala-Asn-His-Tyr-His-Ala-Pro-Asn-Gly-
Gly-Leu-Gly-Pro-Met-Asn-Val-Tyr-Glu-Ser-Lys
25

Peptide 14:

Phe-Arg-Asn-Trp-Ser-Ala-Gly-Tyr-Ala-Asp-Phe-Asp-Arg-Gly-
Thr-Tyr-Val-Ile-Thr-Phe-Ile-Pro-Lys
30

Peptide 15:

Ser-Trp-Asn-Thr-Ala-Pro-Ala-Glu-Val-Lys

Peptide 16 (C-terminal peptide):

35 Gln-Gly-Trp-Ser

Below are shown these sequences aligned to the sequence
of a transglutaminase from *Streptoverticillium* (Kanaji et

al., 1994; Washizu et al., 1994; EP-A-0481 504). Although the two enzymes are homologous they are clearly different as 22% (62 out of 279) of the residues sequenced from the *Streptomyces lydicus* transglutaminase differ from the corresponding residue in the *Streptoverticillium* transglutaminase. It should be stressed that many of the substitutions found are non-conservative - e.g. Asp1Ala, Pro19Ala, Pro22Ala, Ser23Tyr, Tyr24Gly, Glu28Thr, Thr29Val, Arg48Ile, Lys49Gln, Ser84Phe, Lys95Glu, Ser101-Pro, Gly102Asn, Arg105Gln, Gln124Lys, Lys152Thr, Gly157-Lys, Asn163Tyr, Pro169Asn, His188Tyr, Arg208Gln, Ser209Arg, Ala226Asp, Pro227Gln, Ala287Pro, His289Asn, Glu300Ala, Asp324Ala, Lys325Glu and Pro331Ser. The first mentioned residue is the one found in the transglutaminase from *Streptoverticillium* and the second residue is the one found in the transglutaminase from *Streptomyces lydicus*:

Alignment of the peptide sequences obtained from *Streptomyces lydicus* transglutaminase to the amino acid sequence of *Streptoverticillium* transglutaminase:

Upper sequence: *Streptomyces lydicus* transglutaminase

Lower sequence: *Streptoverticillium* transglutaminase

Divergence: 62 out of 279 residues sequenced (22%)

Differences are marked with an asterisk (*)

```

** *          *   *   ***   *          *   **
AADERVTPPA EPLNRMPDAY RAYGGRATTV VNNYIRKWQQ VYAHRDGIQQ
1 DSDDRVTTPA EPLDRMPDPY RPSYGRAETV VNNYIRKWQQ VYSHRDGRKQ
35
                                *   **   ** * *
QMTTEEQRE                      L AFAFFDENK   SDLENSRPR
51 QMTTEEQREWL SYGCVGVTVWV NSGQYPTNRL AFASFDEDRF KNELKNGRPR

```

51

```

      ** *      **      *      **      ** *
PNETQAEFEG RIVK      GFK      ALDSA HDEGTYIDNL
101 SGETRAEFEG RVAKESFDEE KGFQRRAREVA SVMNRALENA HDESAYLDNL

5      *      *      * * *      *      *      *
KTELANKNDA LRYEDGRSNF YSALRNTPSF KERDGGNYDP SK AVVYSK
151 KKELANGNDA LRNEDARSPF YSALRNTPSF KERNGGNHDP SRMKAVIYSK

      *** *      **      *      ** *
10      HFWSGQDQRG SSDK YGDP DAFRPDQGTG LVDMSKDRNI PRSPAQPGES
201 HFWSGQDRSS SADKRKYGDP DAFRPAPGTG LVDMSRDRNI PRSPTSPGEG

      *      * *      * * *      *
WVNFDYGWFG AQ      T IWTHANHYHA PNGGLGPMNV YESKFRNWSA
15 251 FVNFDYGWFG AQTEADADKT VWITHGNHYHA PNGSLGAMHV YESKFRNWSE

      *      *      **      *
GYADFDRGTY VITFIPKSWN TAPAEVKQGW S
301 GYSDFDRGAY VITFIPKSWN TAPDKVKQGW P      331
20

```

EXAMPLE 11

Structural characterization of the Ca^{2+} -independent trans-
25 glutaminase from *Streptomyces platensis*

Structural characterization of the transglutaminase was carried out on an aliquot of highly purified enzyme (4 ml; $A_{280} = 0.74$). The material was lyophilized and redissolved
 30 in 350 μl 6M guanidinium chloride, 0.3 M Tris-HCl, pH 8.3 and denatured overnight at 37 °C. The solution was added 5 μl 0.1 M DTT and incubated for 4 h at room temperature before addition of 25 μl 0.5 M freshly prepared ICH_2COOH . The reduced and S-carboxymethylated sample was desalted using a
 35 NAP5 column (Pharmacia) equilibrated and eluted with 20 mM NH_4HCO_3 . The sample was lyophilized and redissolved in 500 μl 20 mM NH_4HCO_3 .

Of the S-carboxymethylated transglutaminase 200 μ l was added 20 μ g of lysine-specific protease (*Achromobacter* protease I) and degraded for 16 h at 37 °C while another 200 μ l was added 2 μ g of the Asp-N protease from *Pseudomonas* 5 *fragi* and degraded for 16 h at 37 °C. The resulting peptides were fractionated using reversed phase HPLC on a Vydac C18 column eluted with a linear gradient of 80% 2-propanol in 0.1% TFA. Selected peptide fractions were subjected to repurification using reversed phase HPLC on another Vydac 10 C18 column eluted with linear gradients of 80% acetonitrile in 0.1% TFA.

N-terminal amino acid sequencing of the intact transglutaminase as well as sequencing of the purified peptides were 15 done in an Applied Biosystems 473A protein sequencer operated according to the manufacturers instructions.

The sequences obtained are shown in the following:

20 (Xaa designates unidentified residues while Asx designates positions where it could not be determined whether Asp or Asn were present).

N-terminal sequence:

25 Ala-Ala-Asp-Asp-Arg-Val-Thr-Pro-Pro-Ala-Glu-

Peptide 1:

Asp-Asp-Arg-Val-Thr-Pro-Pro-Ala-Glu-Pro-Leu-Asn-Arg-Met-

30 *Peptide 2:*

Ala-Glu-Phe-Glu-Gly-Arg-Ile-Ala-Lys-Gly-Xaa-Phe-

Peptide 3:

35 Asp-Ala-Phe-Arg-Gly-Phe-Lys-Arg-Ala-Arg-Glu-Val-Ala-

Peptide 4:

Asp-His-Leu-Lys-Thr-Glu-Leu-Ala-Asn-Lys-

Peptide 5:

Asp-Ser-Arg-Ser-Ser-Phe-Tyr-Ser-Ala-Leu-Arg-Asn-Thr-Pro-
Ser-Phe-Lys-Glu-Arg

5 *Peptide 6:*

Asp-Pro-Ser-Lys-Met-Lys-Ala-Val-Val-Tyr-Ser-Lys-His-Phe-
Trp-Ser-Gly-Gln

Peptide 7:

10 Asp-Lys-Arg-Lys-Tyr-Gly-Asp-Pro

Peptide 8:

Asp-Tyr-Gly-Trp-Phe-Gly-Ala-Gln-Ala-Glu-

15 *Peptide 9:*

Asp-Lys-Thr-Val-Trp-Thr-His-Ala-Asx-His-Tyr-His-Ala-Pro-
Asx-Gly-Gly-Met-Gly-Pro-Met-Asx-Val-

Peptide 10:

20 Glu-Ser-Lys-Phe-Arg-Asn-Trp-Ser-Ala-Gly-Tyr-Ala

Peptide 11:

Asp-Arg-Gly-Ala-Tyr-Val-Ile-Thr-Phe-Ile-Pro-Lys-Ser-Trp-
Asn-Thr-Ala

25

Peptide 12:

Phe-Phe-Asp-Glu-Asn-Lys

Peptide 13:

30 Arg-Ala-Arg-Glu-Val-Ala-Ser-Val-Met-Asn-Lys

Peptide 14:

Ala-Leu-Asp-Ser-Ala-His-Asp-Glu-Gly-Thr-Tyr-Ile-Asp-His-
Leu-Lys

35

Peptide 15:

Thr-Glu-Leu-Ala-Asn-Lys

Peptide 16:

Ala-Leu-Arg-Asn-Thr-Pro-Ser-Phe-

Peptide 17:

5 Xaa-Xaa-Asp-Gly-Gly-Asn-Tyr-Asp-Pro-Ser-Lys

Peptide 18:

Ala-Val-Val-Tyr-Ser-Lys

10 *Peptide 19:*

His-Phe-Trp-Ser-Gly-Gln-Asp-Pro-Arg-Gly-Ser-Ser-Asp-Lys

Peptide 20:

Tyr-Gly-Asp-Pro-Asp-Ala-Phe-Arg-Pro-Asp-Gln-Gly-Thr-Gly-
15 Leu-Val-Asp-Met-Ser-Arg-Asp-Arg-Asn-Ile-Pro-Arg-Ser-Pro-
Ala-Lys

Peptide 21:

Pro-Gly-Glu-Pro-Phe-Val-Asn-Phe-Asp-Tyr-Gly-Trp-Phe-Gly-
20 Ala-Gln-Ala-Glu-Ala-Asp-Ala-Asp-Lys

Peptide 22:

Thr-Val-Trp-Thr-His-Ala-Asn-

25 *Peptide 23:*

Asn-Trp-Ser-Ala-Gly-Tyr-Ala-Asp-Phe-Asp-Arg-Gly-Ala-Tyr-
Val-Ile-Thr-Phe-Ile-Pro-Lys

Peptide 24:

30 Ser-Trp-Asn-Thr-Ala-Pro-Ala-Glu-Val-Lys

Peptide 25 (C-terminal peptide):

Gln-Gly-Trp-Pro

35 Below the combined sequences are aligned to the sequence of
a transglutaminase from *Streptovercillium* (Kanaji et al.,
1994; Washizu et al., 1994; EP-A-0 481 504). Although the
two enzymes are homologous they are clearly different as

19% (46 out of 240) of the residues sequenced from the *Streptomyces platensis* transglutaminase differ from the corresponding residue in the *Streptoverticillium* transglutaminase. It should be stressed that many of the substitutions found are non-conservative - e.g. Asp1Ala, Ser84Phe, Glu115Gly, Glu119Ala, Glu120Phe, Gln124Lys, Asn149His, Lys152Thr, Gly157Lys, Pro169Ser, His188Tyr, Arg208Pro, Ser209Arg, Ala226Asp, Pro227Gln, Ser246Lys, Gly250Pro, Ala287Pro, His289Asx, Glu300Ala, Asp324Ala and Lys325Glu.

The first mentioned residue is the one found in the transglutaminase from *Streptoverticillium* and the second residue is the one found in the transglutaminase from *Streptomyces platensis*:

Alignment of the combined peptide sequences obtained from *Streptomyces platensis* transglutaminase to the amino acid sequence of *Streptoverticillium* transglutaminase:

X designates an unidentified residue whereas B designates Asx.

20

Upper sequence: *Streptomyces platensis* transglutaminase

Lower sequence: *Streptoverticillium* transglutaminase

25

Divergence: 46 out of 240 residues sequenced (19%)
Differences are marked with an asterisk (*)

30

** *

AADDRVTPPA EPLNRM

1 DSDDRVTTPPA EPLDRMPDPY RPSYGRAETV VNNYIRKWQQ VYSHRDGRKQ

35

* **

FFDENK

51 QMTEEQREWL SYGCVGVTVW NSGQYPTNRL AFASFDEDRF KNELKNGRPR

56

```

      * *   * * *   *           *   **       ** * . *
      AEFEG RIAKGXFDAF RGFKRAREVA SVMNKALDSA HDEGTYIDHL
101 SGETRAEFEG RVAKESFDEE KGFQRAREVA SVMNRALENA HDESAYLDNL

5      *   *           *   *           *   *   *   *
      KTELANK           DSRSSF YSALRNTPSF KERDGGNYDP SKMKAVVYSK
151 KKELANGNDA LRNEDARSPF YSALRNTPSF KERNGGNHDP SRMKAVIYSK

      *** *           **           **       *
10      HFWSGQDPRG SSDKRKYGDP DAFRPDQGTG LVDMSRDRNI PRSPAKPGEP
201 HFWSGQDRSS SADKRKYGDP DAFRPAPGTG LVDMSRDRNI PRSPTSPGEG

      *           *           ** * *           *
      FVNFDYGWFG AQAEADADKT VWTHANHYHA PBGGMGPMBV ESKFRNWSA
15 251 FVNFDYGWFG AQTEADADKT VWTHGNHYHA PNGSLGAMHV YESKFRNWSE

      *           **
      GYADFDRGAY VITFIPKSWN TAPAEVKQGW P
301 GYSDFDRGAY VITFIPKSWN TAPDKVKQGW P   331
20

```

EXAMPLE 12

Streptomyces lydicus transglutaminase - pH and tempera-
25 ture optimum and immunological cross-reactivity with
transglutaminase from *Streptomyces mobaraense*

Enzyme assays:**30 Putrescine assay:**

The putrescine assay was in principle performed according
to Lorand et al. (1972).

The reaction mixture contained: 50 nmoles of [¹⁴C]-putre-
35 scine (4.03 GBq/mmol; Amersham), 6 mg of α-casein
(dephosphorylated, Sigma no. C-8032), 5 μmoles of glutat-
hione, and 5-10 μg of TGase made up to 1 ml with 0.2 M
Tris-HCl, pH 7.9 or 40 mM Britton-Robinson buffer at the

relevant pH. The incubations were performed at ambient temperature. Aliquots of 30 μ l were withdrawn after 1 and 2 h, respectively, and spotted onto Whatman 3 MM filters (D = 2 cm). The filters were immediately put into a basket submerged in ice-cold 10% TCA and washed for 20 min. Following the first wash the filters were washed three times with ice-cold 5% TCA, two times with ice-cold acetone. In each washing step there should be at least 5 ml of washing solution per filter. The filters were dried, put into counting vials containing 8 ml of scintillation fluid (Optiphase, Wallac) and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. Each determination was performed in triplicate.

Hydroxamate assay:

The hydroxamate assay was in principle performed as described by Folk and Cole (1965).

The stop reagent was made of equal volumes of 15% acetic acid, 5% FeCl_3 , and 2.5 N HCl.

20

The reaction mixture contained: 5 μ moles of glutathione, 100 μ moles of hydroxylamine chloride, 30 μ moles of CBZ-Gln-Gly and 0.1 mg TGase made up to 1 ml with 40 mM Britton-Robinson buffer, pH 7.5. The incubations were performed at different temperatures and stopped after 20 min of incubation by addition of an equal volume of stop reagent. The absorbance at 490 nm was measured in an UV_{max} kinetic microplate reader.

30 The temperature optimum for the *S. lydicus* TGase was measured in the hydroxamate assay and optimum was found to be 50 °C. The results were:

58

Temp (°C)	Relative activity (%)
30	30
40	75
45	90
50	100
55	75
60	20
70	10

10

The pH profile for the *S. lydicus* TGase was determined in the putrescine assay varying pH from 6 to 9. Optimum was found to be around pH 8. The results were:

15

pH	Relative activity (%)
6.0	6
6.5	12
7.0	21
7.5	40
8.0	57
8.5	83
9.0	100

20

25 The TGase from *S. lydicus* was analyzed for immunological cross-reactivity with the TGase from *Streptovercillium mobaraense*. A polyclonal antibody was raised against the pure *S. mobaraense* enzyme and using an Ouchterlony immunodiffusion assay there was found to be no cross-reacti-

vity between the TGases from *S. lydicus* and *S. mobaraense*.

Experimental:

- 5 Four rabbits were immunized with the pure TGase from *S. mobaraense* according to standard procedures. The anti-serum from all four rabbits was pooled and the antibody was purified on a HiTrap Protein G column from Pharmacia following the recommended procedure. The purified anti-
10 body was used in an Ouchterlony immunodiffusion (1% agarose in 0.1 M Tris-HCl) using the pure TGases from *S. mobaraense* and *S. lydicus* as antigens.

15 **EXAMPLE 13**

Fermentation and production of TGase from *Streptomyces lydicus*, NRRL B-3446 (former *Streptomyces libani*)

- 20 The strain was grown in a 2 liter fermentation vessel supplied with a magnetic coupled stirrer drive, pH- and temperature control and a peristaltic pump to add the carbon source at fixed rates. After growth for 3 days on a YPG agar slant at 30°C a lump of the mycelium was inocu-
25 lated in to a 500 ml shakeflask containing a YPD-broth (2% yeast extract, 1 % Bacto peptone, 6 % glucose) and propagated for 24 hours at 250 rpm and 30°C. 100 ml of this culture was used to inoculate the fermentor already containing 1,3 liter broth with the following ingredients:

30

Yeast extract, 50 %:	60 g
Amicase:	30 g
MgS ₇ SOO ₄ .7H ₂ O :	3 g
K ₂ SO ₄ :	4 g
35 Trace metals :	3 ml
Vitamin I :	1.5 ml
Vitamin II :	1.5 ml
Pluronic(antifoam):	3 ml

Volume adjusted to 1.3 liters with tap water. pH adjusted to 7.0 before sterilization in an autoclave at 121°C for 60 minutes. Also a glucose solution was separately made in a 1 liter flask containing 250 g of glucose, 1H₂O and 0.25 grams of citric acid in tapwater. Volume adjusted to 500 ml before autoclaving as above.

Biomass growth and enzyme formation:

After inoculation of the culture as described above the glucose solution was fed at a constant rate (9 g/h) to the fermentor over the next 17 hours. A peristaltic Watson-Marlow pump was used. Also sterile filtered air was sparged into the fermentor at the bottom drive at a rate of 1.4 liter/minute and this rate was kept throughout the fermentation. The stirrer speed was at the beginning set to 300 rpm, but automatically coupled to the dissolved oxygen tension signal and a setpoint of 10% DOT and therefore running close to the maximum value of 1100 rpm after 17 hours. The glucose feed rate was now increased to 14.5 grams/hour, which was kept over the next 24 hours and finishing the glucose reservoir. During this period stirrer speed was at the maximum (1150 rpm) and DOT close to zero. A surplus of glucose was also present and this was diminished to ~0.1% glucose over the next 7 hours. Temperature was controlled at 30.0 +/- 0.1 °C and pH to 7.00 +/- 0.05 by addition of diluted ammonia in water.

After these 48 hours of growth the culture, with a biomass of 45 g of dry biomass per liter, was harvested and the very viscous mycelium nearly quantitatively removed by addition of 500 ml of tap water. The suspension was stored in the cold (4°C) for 3 days. The supernatant was removed after centrifugation 30 minutes at 4000 rpm. The precipitate was diluted to the original volume in tap water, suspended and again centrifuged, now for 45 minutes. Enzyme concentrations in the two supernatants were determined by the hydroxamate assay.

Yields are shown in the table below:

	Quantity, grams	Yield of TGase, mg/l	Yield of TGase, mg
Diluted broth	1993		
5 Supernatant I	1536	120	184
SupernatantII	1675	50	84
Total			268

This yield corresponds to ~180 mg/l undiluted broth.

10

This should be compared with the prior art yields of transglutaminase reported to be not higher than about 2.47 units/ml in the hydroxamate assay, see US 5,252,469.

15 Trace metal solution.

	Conc.HCl	5 ml
	ZnCl ₂	3.4 g
	FeCl ₃ .6H ₂ O	27 g
20	MnCl ₂ .4H ₂ O	9.55 g
	CuSO ₄ .5H ₂ O	1.1 g
	CoCl ₂	1.29 g
	H ₃ BO ₃	0.31 g
	(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	0.1 g
25	Distilled water at	1000 ml

Vitamin I

	Biotin	1,25 g
30	Thiamin	20 g
	D-calciumpanthotenate	250 g
	Myoinositol	500 g
	Cholinchloride	500 g
	Pyridoxin	16 g

62

Niacinamide	12	g
Folicacid	2	g
Destilled water at	10	l

5 Vitamin II

Riboflavine	4	g
Destilled water at	10	l

10

EXAMPLE 14**Viscosity increase in Na-caseinate solution**

- 15 A solution of Na-caseinate (Miprodan 30, MD Foods, Denmark) was prepared containing 9% protein. pH was adjusted to 7.0 using NaOH.

Viscosity was measured using the Sofraser MIVI 2000 viscosimeter. Viscosity reading is given as mV setting the set point to 0 mV when measuring without enzyme addition.

The experiment compared two transglutaminases:

1. Commercial transglutaminase (Ajinomoto TG-K) which is formulated with dextrin 24%, Ca-lactate 75% and enzyme 1%.
2. Freeze dried enzyme preparation from *Streptomyces lydicus*, NRRL B-3446 (former *Streptomyces libani*) fermentation.

30

Activity of both enzymes are measured by the hydroxamate assay as described in EP 0379606 A1. Based upon this the dosage for both enzymes were 0,36mg enzyme for a 5 ml substrate solution.

35

The experiment was carried out twice at 50°C and 55°C, respectively, and the results are shown in the tables below.

Measurement (mV) at 50°C:

	Time, minutes	TG-K	Streptomyces lydicus
	0	0	0
5	6	110	147
	11	64	161
	15	70	173
	20	130	300
	25	240	439
10	30	323	561
	35	333	612
	40	390	728
	45	514	833
	50	813	947
15	55	1142	992
	60	1374	1040
	65	1416	1097
	70	1230	1160
20	75	1269	1196

Measurement (mV) at 55°C:

Time, minutes	TG-K	Strepto- mycesly- dicus
0	0	0
5	108	55
10	133	175
15	233	289
25	329	468
30	399	602
40	477	691
50	661	784
60	596	873
70	745	895
75	720	892

15

Both enzymes show activity at 50°C and 55°C. The results shows that the activity of *Streptomyces lydicus*, NRRL B-3446 (former *Streptomyces libani*) transglutaminase is higher at 55°C compared to the TG-K transglutaminase indicating a higher temperature optimum and/or higher thermostability for the *Streptomyces lydicus*, NRRL B-3446 (former *Streptomyces libani*) enzyme.

20

25 EXAMPLE 15

Gene encoding the transglutaminase from *Streptomyces lydicus*

MATERIALS AND METHODS

Donor organism:

DNA was isolated from *Streptomyces lydicus*, NRRL B-3446.

- 5 The used host was *E. coli* SJ2 (Diderichsen, B. et al., (1990)).

- Plasmid:** The gene bank vector was pSJ1678 which is further disclosed in WO94/19454 which is hereby incorporated by reference. The cloning vector was pPL1759.
- 10

- Chromosomal DNA from *Streptomyces lydicus*, NRRL B-3446, was partial digested with the restriction enzyme Sau3A1. The fragments were cloned into the BamHI sites of a cloning vector pSJ1678, cf. figure 1 and WO 95/01425, and transformed into *Escherichia coli* SJ2 (Diderichsen, B. et al., (1990)), thereby creating a gene library of *S.lydicus*.
- 15

- 20 From the protein sequence made by sequencing the *S.lydicus* transglutaminase protein two PCR primers were predicted. A primer containing a PstI site and the predicted 30 bases of the 5'-terminal (primer 7854) and a primer containing a restriction enzyme HindIII recognition sequence and 30 bases complementary to the predicted transglutaminase 3' sequence (primer 7855) of the mature transglutaminase gene from *S.lydicus* was prepared:
- 25

7854 :

- 30 5'-CCTCATTCTGCAGCAGCGGCGGCAGCCGACGAAAGGGTCACCCCTCCCGCC-3'

7855 :

5'-GCGCGAAGCTTCACGACCAGCCCTGCTTTACCTCGGCGGGGGC-3'

- 35 2-4 mg Chromosomal DNA from *S.lydicus* was used as template in a PCR reaction (20 cycles) using the primers 7854 and 7855 and Super Taq DNA polymerase and following the manufacturer's instructions (Super Taq™ DNA polymerase-

se/PCR buffer, HT BIOTECHNOLOGY LTD).

A PCR fragment corresponding to the expected size of the transglutaminase from *S.lydicus* was recovered from an agarose gel and digested with the restriction enzymes HindIII and PstI.

The plasmid pPL1759, see figure 2 and Hansen, C. (1992), was digested with the restriction enzymes PstI-HindIII and the large vector fragment was ligated to the PCR fragment. Ligation mixture was transformed into *Bacillus subtilis* DN1885 (P.L.Jørgensen et al., (1990)).

Selection for transformants and reisolation of those was performed on LBPG media with 10 µg Kanamycin/ml. DNA analysis of the plasmids from those clones using a DNA sequencing Kit (SEQUENASE™ (United States Biochemicals)) showed the expected sequence (SEQ ID No. 1) of the mature transglutaminase encoding region when it was translated and compared to the partial sequenced transglutaminase protein of *S.lydicus*. This plasmid was termed pJA243 and a *B. subtilis* DN1885 strain harbouring this plasmid was termed JA243. A plasmid map of pJA243 is shown in figure 3.

25

The PstI-HindIII fragment of pJA243 was used as template for making a radioactive labeled probe using the Nick Translation Kit™ as described by the manufacturer (code N.5500 from Amersham). This radioactive probe was used for colony hybridization to the gene bank of *S.lydicus*. To find the native transglutaminase gene a positive clone was isolated. This bacterium contained a fragment inserted in plasmid pSJ1678. The cloned DNA could be amplified with the primers 7854 and 7855 giving a fragment of the correct size. The clone was denoted JA260 and deposited under the Budapest Treaty on 23 August 1995 as DSM 10175 (*E. coli*).

EXAMPLE 16**Inhibition of bacterial TGases with PMSF (Phenyl methyl sulfonyl fluoride)**

5

A putrescine control assay was performed to investigate whether TGases from different bacterial strains are sensitive to PMSF (phenyl methyl sulfonyl fluoride). The purified transglutaminases from *S. lydicus* and *S. mobara-*
 10 *ense* was found to be insensitive to PMSF.

The assay was run under optimized conditions (see examples 1-3), i.e. 1 h incubation at 30°C, pH 8.5 (2% α -casein + EDTA in 100 mM modified Britton-Robinson-buffer
 15 0.1 M, pH 8.5). Final concentration of PMSF was 1.6 mM. As control an assay including propanol was included: propanol is the solvent of PMSF. This assay ensures that solvent effects are excluded. The assay contained 0.9 M 2-propanol i.e. the same concentration as in PMSF containing assay (addition of 8 μ l propanol from 13 M instead
 20 of 8 μ l PMSF in 2-propanol).

Results:

25	Strain	Relative activity (%)	
		-PMSF	+PMSF
	<i>Bacteridium</i>		
	(DSM 10093)	100	75
	<i>Bacteridium</i>		
30	(CBS 495.74)	100	75
	<i>Moo5A10</i>	100	75
	<i>Bacillus badius</i>	100	62
	<i>Bacillus firmus</i>	100	55
	<i>Bacillus mycoides</i>	100	35
35	<i>B. aneurinolyticus</i>	100	47
	<i>S. lydicus</i>	100	175

TGase activity of all strains investigated except *S. ly-*

dicus was inhibited by the addition of PMSF: *Bacillus* *ba-*
dus, *Bacillus firmus*, *Bacillus mycoides*, *Bacillus*
aneurinolyticus, strain Moo5A10, *Bacteridium* (DSM10093)
and *Bacteridium* (CBS 495.74).

5

This result is in contrast to the TGase from *Streptomyces*
lydicus and the known microbial transglutaminases. This
result implies that the PMSF-sensitive TGases listed abo-
ve possess different catalytic active sites than the TGA-
10 ses from *Streptomyces* and all other known TGases (inclu-
ding Factor XIII).

EXAMPLE 17

15 Gelling of skimmilk with bacterial TGases

Experimental:

150 μ l of skimmilk solutions (15 and 20 %) and 50 μ l of
supernatant (i.e. different TGase activities) were incu-
20 bated in Eppendorf Tubes at 30°C while shaking with 700
rpm on a thermostated shaker.

Assays were visibly controlled every hour and after over
night incubation (about 18 h).

25 Samples used were centricon treated (in order to elimi-
nate Ca^{2+} -ions): corresponding samples (1ml) were concen-
trated in a 10kDa Centricon tube (no TGase activity in
the filtrate), resuspended with Ca^{2+} -free Tris-buffer
(0.1 M, pH 7.5) up to 1 ml, centrifuged again and concen-
30 trated by resuspending the retentate in 0.25 ml of Tris
buffer.

Controls: As some of the media used contain high concen-
trations of Ca^{2+} (Medium H: 34 mM; Medium K and Q: 3.4 mM;
35 Medium L: 13.6 mM) controls were run to check Ca^{2+} -depen-
dent (Tgase-independent) effects. The media were treated
as described for the samples with centricon tubes.

Results:

All strains investigated showed a gelling of 15 and/or 20% skimmilk:

Bacteridium (2 strains), *Bacillus firmus*, *Bacillus mycoi-*
5 *des*, *Bacillus badius*, *Bacillus aneurinolytik*, strain
Moo5A10 and *Rothia dentocariosa*. *Bacillus mycoides* and
Bacillus aneurinolyticus showed positive effects after 60
minutes of incubation, all others were positive after
over night incubation.

REFERENCES

- Klein et al., Journal of Bacteriology, Vol. 174, pages 2599-2605, (1992).
- 5
- US 5,156,956 (Motoki et al.)
- US 5,252,469 (Andou et al.).
- 10 Kaempfer et al., Journal of General Microbiology, Vol. 137, pages 1831-1892, (1991).
- Ochi et al., International Journal of Systematic Bacteriology, Vol. 44, pages 285-292, (1994).
- 15
- Williams et al., Journal of General Microbiology, Vol. 129, pp. 1743-1813.
- Washizu et al., Bioscience, Biotechnology and Biochemistry Vol. 58, pages 82-87, (1994).
- 20
- Tahakana et al., *ibid.* Vol. 58, pages 88-92.
- EP-0 481 504 A1 (Takagi et al.).
- 25
- US 5,252,469.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab., Cold Spring Harbor, NY.
- 30
- Ford et al., Protein Expression and Purification 2: 95-107, 1991.
- 35
- Cunningham and Wells, Science 244, 1081-1085, (1989).
- de Vos et al., Science 255: 306-312, (1992).

Smith et al., J. Mol. Biol. 224: 899-904, 1992.

Wlodaver et al., FEBS Lett. 309: 59-64, 1992.

- 5 N. Axelsen et al., A Manual of Quantitative Immuno-electrophoresis, Blackwell Scientific Publications, 1973, Chapters 2,3,4 and 23.

Needleman, S.B. and Wunsch, C.D., *Journal of Molecular*
10 *Biology*, 48: 443-453, 1970

WO 94/14953

US 4,683,202

15

Saiki, R.K. et al., (1988), *Science* **239**, p. 487-491.

Beaucage and Caruthers, (1981), *Tetrahedron Letters* **22**,
p. 1859-1869.

20

Matthes et al., (1984), *EMBO Journal* **3**, p. 801-805.

EP-A-0 238 023

- 25 A. Johnstone and R. Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31).

O. Ouchterlony in: *Handbook of Experimental Immunology*
30 (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706.

WO 95/01425

- 35 Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B.R., Sjøholm, C., *Journal of Bacteriology*, Vol. 172, No. 8, p. 4315-4321, (1990).

WO94/19454

Hansen, C. (1992) PhD Thesis, The Technical University of Denmark

5

P.L.Jørgensen et al. , *Gene* (1990) **96**, 37-41

Folk, J. E. & Cole, P. W. (1966), *J. Biol. Chem.* **241**,
5518-5525

10

EP-A-0 481 504

Curtis, C. G. & Lorand, L. (1976), *Methods in Enzymology*
45, 177-191

15

Kanaji et al., *J. Biol. Chem.* **268**, 11565-11572, 1994.

Lorand, L., Campbell-Wilkes, L.K., and Cooperstein, L.
(1972), *Anal. Biochem.*, **50**, 623-631

20

Folk, J.E., and Cole, P.W. (1965) *J. Biol. Chem.*, **240**,
2951

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: Novo Nordisk A/S
- (B) STREET: Novo Alle
- (C) CITY: DK-2880 Bagsvaerd
- 10 (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2880
- (G) TELEPHONE: +45 44 44 88 88
- (H) TELEFAX: +45 44 49 32 56
- (I) TELEX: 37304

15

(ii) TITLE OF INVENTION: TITLE

(iii) NUMBER OF SEQUENCES: 2

20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version
- 25 #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 993 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

74

(A) ORGANISM: *Streptomyces lydicus*

(C) INDIVIDUAL ISOLATE: NRRL B-3446

(ix) FEATURE:

5

(A) NAME/KEY: CDS

(B) LOCATION: 1..993

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```

10  GCA GCC GAC GAA AGG GTC ACC CCT CCC GCC GAG CCG CTC AAC CGG ATC  48
    Ala Ala Asp Glu Arg Val Thr Pro Pro Ala Glu Pro Leu Asn Arg Met
      1             5             10             15

    CCT GAC GCG TAC CGG GCC TAC GGA GGT AGG GCC ACT ACG GTC GTC AAC  96
15  Pro Asp Ala Tyr Arg Ala Tyr Gly Gly Arg Ala Thr Thr Val Val Asn
      20             25             30

    AAC TAC ATA CGC AAG TGG CAG CAG GTC TAC AGT CAC CGC GAC GGC ATC 144
    Asn Tyr Ile Arg Lys Trp Gln Gln Val Tyr Ser His Arg Asp Gly Ile
20      35             40             45

    CAA CAG CAA ATG ACC GAA GAG CAG CGA GAA AAG CTG TCC TAC GGC TGC 192
    Gln Gln Gln Met Thr Glu Glu Gln Arg Glu Lys Leu Ser Tyr Gly Cys
      50             55             60

25  GTC GGC ATC ACC TGG GTC AAT TCG GGC CCC TAC CCG ACG AAT AAA TTG 240
    Val Gly Ile Thr Trp Val Asn Ser Gly Pro Tyr Pro Thr Asn Lys Leu
      65             70             75             80

30  GCG TTC GCG TTC TTC GAC GAG AAC AAG TAC AAG AGT GAC CTG GAA AAC 288
    Ala Phe Ala Phe Phe Asp Glu Asn Lys Tyr Lys Ser Asp Leu Glu Asn
      85             90             95

    AGC AGG CCA CGC CCC AAT GAG ACG CAA GCC GAG TTT GAG GGG CGC ATC 336
35  Ser Arg Pro Arg Pro Asn Glu Thr Gln Ala Glu Phe Glu Gly Arg Ile
      100            105            110

    GTC AAG GAC AGT TTC GAC GAG GGG AAG GGT TTC AAG CGG GCG CGT GAT 384
    Val Lys Asp Ser Phe Asp Glu Gly Lys Gly Phe Lys Arg Ala Arg Asp
40      115            120            125

    GTG GCG TCC GTC ATG AAC AAG GCC CTG GAT AGT GCG CAC GAC GAG GGG 432
    Val Ala Ser Val Met Asn Lys Ala Leu Asp Ser Ala His Asp Glu Gly
      130            135            140

```

75

ACT TAC ATC GAC AAC CTC AAG ACG GAG CTC GCG AAC AAA AAT GAC GCT 480
 Thr Tyr Ile Asp Asn Leu Lys Thr Glu Leu Ala Asn Lys Asn Asp Ala
 145 150 155 160

5 CTG CGC TAC GAG GAC GGT CGC TCG AAC TTT TAC TCG GCG CTG AGG AAT 528
 Leu Arg Tyr Glu Asp Gly Arg Ser Asn Phe Tyr Ser Ala Leu Arg Asn
 165 170 175

ACG CCG TCC TTC AAG GAA AGG GAT GGA GGT AAC TAC GAC CCA TCC AAG 576
 10 Thr Pro Ser Phe Lys Glu Arg Asp Gly Gly Asn Tyr Asp Pro Ser Lys
 180 185 190

ATG AAG GCG GTG GTC TAC TCG AAA CAC TTC TGG AGC GGG CAG GAC CAG 624
 15 Met Lys Ala Val Val Tyr Ser Lys His Phe Trp Ser Gly Gln Asp Gln
 195 200 205

CGG GGC TCC TCT GAC AAG AGG AAG TAC GGC GAC CCG GAT GCC TTC CGC 672
 Arg Gly Ser Ser Asp Lys Arg Lys Tyr Gly Asp Pro Asp Ala Phe Arg
 210 215 220

20 CCC GAC CAG GGC ACA GGC CTG GTA GAC ATG TCG AAG GAC AGG AAT ATT 720
 Pro Asp Gln Gly Thr Gly Leu Val Asp Met Ser Lys Asp Arg Asn Ile
 225 230 235 240

25 CCG CGC AGT CCC GCC CAA CCT GGC GAA AGT TGG GTC AAT TTC GAC TAC 768
 Pro Arg Ser Pro Ala Gln Pro Gly Glu Ser Trp Val Asn Phe Asp Tyr
 245 250 255

GGC TGG TTT GGG GCT CAG ACG GAA TCG GAC GCC GAC AAA ACC ATA TGG 816
 30 Gly Trp Phe Gly Ala Gln Thr Glu Ser Asp Ala Asp Lys Thr Ile Trp
 260 265 270

ACC CAC GCC AAC CAC TAT CAC GCG CCC AAC GGC GGC CTG GGC CCC ATG 864
 35 Thr His Ala Asn His Tyr His Ala Pro Asn Gly Gly Leu Gly Pro Met
 275 280 285

AAC GTA TAT GAG AGC AAG TTC CGG AAC TGG TCT GCC GGG TAC GCG GAT 912
 Asn Val Tyr Glu Ser Lys Phe Arg Asn Trp Ser Ala Gly Tyr Ala Asp
 290 295 300

40 TTC GAC CGC GGA ACC TAC GTC ATC ACG TTC ATA CCC AAG AGC TGG AAC 960
 Phe Asp Arg Gly Thr Tyr Val Ile Thr Phe Ile Pro Lys Ser Trp Asn
 305 310 315 320

45

76

ACC GCC CCC GCC GAG GTA AAG CAG GGC TGG TCG
 Thr Ala Pro Ala Glu Val Lys Gln Gly Trp Ser
 325 330

993

5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 331 amino acids

10

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

15

Ala Ala Asp Glu Arg Val Thr Pro Pro Ala Glu Pro Leu Asn Arg Met
 1 5 10 15

20

Pro Asp Ala Tyr Arg Ala Tyr Gly Gly Arg Ala Thr Thr Val Val Asn
 20 25 30

Asn Tyr Ile Arg Lys Trp Gln Gln Val Tyr Ser His Arg Asp Gly Ile
 35 40 45

25

Gln Gln Gln Met Thr Glu Glu Gln Arg Glu Lys Leu Ser Tyr Gly Cys
 50 55 60

Val Gly Ile Thr Trp Val Asn Ser Gly Pro Tyr Pro Thr Asn Lys Leu
 65 70 75 80

30

Ala Phe Ala Phe Phe Asp Glu Asn Lys Tyr Lys Ser Asp Leu Glu Asn
 85 90 95

35

Ser Arg Pro Arg Pro Asn Glu Thr Gln Ala Glu Phe Glu Gly Arg Ile
 100 105 110

Val Lys Asp Ser Phe Asp Glu Gly Lys Gly Phe Lys Arg Ala Arg Asp
 115 120 125

40

Val Ala Ser Val Met Asn Lys Ala Leu Asp Ser Ala His Asp Glu Gly
 130 135 140

77

	Thr Tyr Ile Asp Asn Leu Lys Thr Glu Leu Ala Asn Lys Asn Asp Ala	
	145	150 155 160
5	Leu Arg Tyr Glu Asp Gly Arg Ser Asn Phe Tyr Ser Ala Leu Arg Asn	
	165	170 175
	Thr Pro Ser Phe Lys Glu Arg Asp Gly Gly Asn Tyr Asp Pro Ser Lys	
	180	185 190
10	Met Lys Ala Val Val Tyr Ser Lys His Phe Trp Ser Gly Gln Asp Gln	
	195	200 205
	Arg Gly Ser Ser Asp Lys Arg Lys Tyr Gly Asp Pro Asp Ala Phe Arg	
	210	215 220
15	Pro Asp Gln Gly Thr Gly Leu Val Asp Met Ser Lys Asp Arg Asn Ile	
	225	230 235 240
	Pro Arg Ser Pro Ala Gln Pro Gly Glu Ser Trp Val Asn Phe Asp Tyr	
20	245	250 255
	Gly Trp Phe Gly Ala Gln Thr Glu Ser Asp Ala Asp Lys Thr Ile Trp	
	260	265 270
25	Thr His Ala Asn His Tyr His Ala Pro Asn Gly Gly Leu Gly Pro Met	
	275	280 285
	Asn Val Tyr Glu Ser Lys Phe Arg Asn Trp Ser Ala Gly Tyr Ala Asp	
	290	295 300
30	Phe Asp Arg Gly Thr Tyr Val Ile Thr Phe Ile Pro Lys Ser Trp Asn	
	305	310 315 320
	Thr Ala Pro Ala Glu Val Lys Gln Gly Trp Ser	
35	325	330

Applicant's or agent's file
reference number

11-WO, AMJ

International application No

78

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description
on page 6, line 12-16

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-
KULTUREN GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1b, D-38124 Braunschweig, Federal Re-
public of Germany

Date of deposit

23 August 1995 (23.08.95)

Accession Number

DSM 10175

C. ADDITIONAL INDICATIONS (leave blank if not applicable)

This information is continued on an additional sheet ☐

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

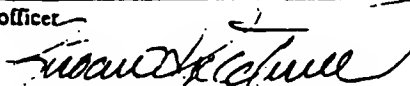
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer



For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

CLAIMS

1. A transglutaminase preparation which is producible by cultivation of a fungus.
- 5 2. The preparation according to claim 1, wherein the fungus is a basidiomycotina, an ascomycotina or a zygomycotina.
- 10 3. The preparation according to claim 2, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the orders *Agaricales*, *Aphylllophorales*, *Ceratobasidiales*, *Auriculariales* and *Nidulariales*.
- 15 4. The preparation according to claim 3, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the families *Tricholomataceae*, *Amanitaceae*, *Agaricaceae*, *Strophariaceae*, *Copri-*
20 *naceae*, *Cortinariaceae*, *Paxillaceae*, *Polyporaceae*, *Coriolaceae*, *Fomitopsidaceae*, *Stereaceae*, *Hymenochaetaceae*, *Lachnocladiaceae*, *Ceratobasidiaceae*, *Auriculariaceae* and *Nidulariaceae*.
- 25 5. The preparation according to claim 4, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the genera *Tricholoma*, *Lyophyllum*, *Armillaria*, *Amanita*, *Agaricus*, *Chamaemyces*, *Stropharia*, *Hypholoma*, *Kuhneromyces*, *Pholiota*, *Coprinus*,
30 *Psathyrella*, *Panaeolus*, *Gymnopilus*, *Hygrophoropsis*, *Pleurotus*, *Pycnoporus*, *Antrodia*, *Trametes*, *Amylostereum*, *Hymenochaete*, *Scytinostroma*, *Rhizoctonia*, *Auricularia* and *Nidula*.
- 35 6. The preparation according to claim 5, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the species *Tricholoma flavovirens* or *Tricholoma myomyces*, *Lyophyllum* sp., *Ar-*

millaria sp., *Amanita virosa*, *Agaricus* sp., *Chamaemyces fracidus*, *Stropharia coerulea*, *Hypholoma fasciculare*, *Kuhneromyces variabilis*, *Pholiota jahnii*, *Coprinus cinereus*, *Coprinus* sp., *Psathyrella condolleana*, *Panaeolus papilionaceus*, *Gymnopilus junonius*, *Hygrophoropsis aurantiaca*, *Pleurotus dryinus*, *Pleurotus* sp., *Pycnoporus cinnabarinus*, *Antrodia serialis*, *Trametes hirsuta*, *Amylostereum chailletii*, *Hymenochaete corticola*, *Scytinostroma portentosum*, *Rhizoctonia solani*, *Auricularia polytricha* and *Nidula* sp..

7. The preparation according to claim 6, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the species *Armillaria* sp., CBS 372.94; *Coprinus cinereus*, IFO 30116; *Psathyrella condolleana*, CBS 628.95; *Panaeolus papilionaceus*, CBS 630.95; *Amylostereum chailletii*, CBS 373.94; and *Hymenochaete corticola*, CBS 371.94.

8. The preparation according to claim 2, wherein the zygomycota is selected from a strain belonging to the order *Mucorales*.

9. The preparation according to claim 8, wherein the fungus is a zygomycotum selected from a strain belonging to the group consisting of the genera *Mucor* and *Cunninghamella*.

10. The preparation according to claim 9, wherein the fungus is a zygomycota selected from a strain belonging to the group consisting of the species *Mucor aligarensis*, preferably ATCC 28928, *Mucor luteus* and *Cunninghamella elegans*, preferably AHU 9445.

11. The preparation according to claim 2, wherein the ascomycetes is selected from a strain belonging to the group consisting of the classes *Discomycetes*, *Pyrenomyces*, *Loculoascomycetes*, and *Plectomycetes*.

12. The preparation according to claim 2 or 11, wherein the ascomycetes is selected from a strain belonging to the group consisting of the orders *Leotiales*, *Xylariales*, *Diaporthales*, *Sordariales*, *Halosphaeriales*, *Hypocreales*,
5 *Dothideales*, *Eurotiales*, and *Ascomycetes* of unknown order.

13. The preparation according to claim 2 or 12, wherein the ascomycetes is selected from a strain belonging to
10 the group consisting of the families *Leotiaceae*, *Xylariaceae*, *Amphisphaeriaceae*, *Valsaceae*, *Chaetomiaceae*, *Lasiosphaeriaceae*, *Halosphaeriaceae*, *Hypocreaceae*, *Pleosporaceae*, *Mycosphaerellaceae*, *Botryosphaeriaceae*, *Sporormiaceae*, *Herpotrichiellaceae*, and *Trichocomataceae*.

15 14. The preparation according to claim 2 or 13, wherein the ascomycetes is selected from a strain belonging to the group consisting of the genera *Dimorphosporum*, *Xylaria*, *Ascotricha*, *Nodulisporium*, *Savoryella*, *Valsa*, *Chaetomium*, *Podospora*, *Halosphaeriopsis*, *Lulworthia*, *Lignincola*, *Fusarium*, *Myrothecium*, *Trichoderma*, *Alternaria*,
20 *Cochliobolus*, *Curvularia*, *Cercospora*, *Cladosporium*, *Botryosphaeria*, *Sporormiella*, *Preussia*, *Carponia*, *Coniothyrium*, *Byssochlamys*, *Talaromyces*, *Neosartorya*, *Warcupella*, *Aspergillus*, *Beauveria*, *Hortea*, *Humicola*, *Monodictys* and *Dendryphiella*.

15. The preparation according to claim 2 or 14, wherein the ascomycetes is selected from a strain belonging to
30 the group consisting of the species *Dimorphosporum disparatrichum*, *Xylaria* sp., *Ascotricha erinacea*, *Nodulisporium* sp., *Savoryella lignicola*, *Valsa pini*, *Chaetomium funiculum*, *Podospora tetraspora*, *Halosphaeriopsis medio-setigera*, *Lulworthia uniseptata*, *Lignincola* sp., *Fusarium*
35 *armeniaceum*, *Fusarium decemcellulare*, *Fusarium dimerum*, *Fusarium merismoides*, *Fusarium redolens*, *Fusarium flocciferum*, *Myrothecium roridum*, *Trichoderma harzianum*, *Alternaria alternata*, *Cochliobolus sativus*, *Curvularia borrei-*

- ae, *Cercospora beticola*, *Cercospora carisis*, *Cercospora fusimaculans*, *Cercospora hayi*, *Cercospora sesami*, *Cercospora traversiana*, *Cladosporium cladosporioides*, *Cladosporium resinae*, *Cladosporium oxysporum*, *Cladosporium sp-*
 5 *haeospermum*, *Botryosphaeria rhodina*, *Sporormiella australis*, *Sporormiella minima*, *Preussia isomera*, *Carponia solliomaris*, *Coniothyrium cerealis*, *Byssochlamys fulva*, *Talaromyces helicus*, *Neosartorya quadricineta*, *Warcupiellea spinulosa*, *Aspergillus foetidus*, *Aspergillus giganteus*,
 10 *Aspergillus heteromorphus*, *Aspergillus puniceus*, *Aspergillus tamarisii*, *Beauveria cylindrospora*, *Beauveria calendonica*, *Hortea werneckii*, *Humicola alopallonella*, *Monodictys pelagica* and *Dendryphiella salina*.
- 15 16. The preparation according to claim 15, wherein the ascomycetes is selected from a strain belonging to the group consisting of the species *Dimorphosporum disparatrichum*, ATCC 24562; *Savoryella lignicola*, CBS 626.95; *Chaetomium funiculum*, ATCC 42779; *Lulworthia*
 20 *uniseptata*, IFO 32137; *Fusarium armeniacum*, IBT 2173; *Fusarium decemcellulare*, CBS 315.73; *Fusarium dimerum*, IBT 1796; *Fusarium merismoides*, ATCC 16561; *Fusarium redolens*, IBT 2058; *Myrothecium roridum*, ATCC 20605; *Trichoderma harzianum*, CBS 223.93; *Alternaria alternata*, CBS
 25 448.94; *Curvularia borreiae*, CBS 859.73; *Cercospora beticola*, ATCC 28056; *Cercospora carisis*, IMI 167.425; *Cercospora fusimaculans*, IMI 167.426; *Cercospora hayi*, IMI 160.414; *Cercospora sesami*, IMI 318.913; *Cercospora traversiana*, IMI 318.080; *Cladosporium resinae*, CBS 174.61;
 30 *Cladosporium sphaeospermum*, CBS 444.94; *Byssochlamys fulva*, AHU 9252; *Talaromyces helicus*, ATCC 10451; *Neosartorya quadricineta*, IBT 11057; *Warcupiellea spinulosa*, NKBC 1495; *Aspergillus foetidus*, CBS 565.65; *Aspergillus giganteus*, CBS 526.65; *Aspergillus heteromorphus*, CBS
 35 117.55; *Aspergillus puniceus*, IAM 13893; *Aspergillus tamarisii*, IBT 3824; *Beauveria cylindrospora*, CBS 719.70; *Beauveria calendonica*, CBS 485.88; *Hortea werneckii*, CBS 446.94; *Monodictys pelagica*, CBS 625.95; and Den-

dryphiella salina, CBS 447.94.

17. The preparation according to any of the claims 1-16 which polymerizes α -casein.

5

18. The preparation according to any of the claims 1-17, wherein the transglutaminase exhibits optimum activity at a temperature of at least 55°C, preferably at least 60°C, more preferably at least 70°C even more preferably at
10 least 80°C, especially at least 90°C.

19. The preparation according to claim 18, wherein the transglutaminase is producible by a strain belonging to the genera *Savoryella*, *Cladosporium*, *Monodictys*, *Hymenochaete* and *Lulworthia*.
15

20. The preparation according to claim 19, wherein the transglutaminase is producible by a strain belonging to the species *Savoryella lignicola*, *Cladosporium sphaeospermum*, *Hymenochaete corticola*, *Monodictys pelagica* and *Lulworthia uniseptata*.
20

21. The preparation according to any of the claims 1-17, wherein the transglutaminase exhibits optimum relative
25 activity at a pH of at least 8.5, preferably at least 9.0.

22. The preparation according to claim 21, wherein the transglutaminase is producible by a strain belonging to
30 the genera *Savoryella*, *Cladosporium*, *Cercospora*, *Hymenochaete*, *Monodictys* and *Lulworthia*.

23. The preparation according to any of the claims 1-22, wherein the transglutaminase activity is inhibited by
35 phenylmethylsulfonylfluoride (PMSF).

24. A transglutaminase preparation which is producible by cultivation of a bacterium, provided that the bacterium

does not belong to Cluster F of *Streptomyces* and related genera.

25. The preparation according to claim 24, wherein the
5 bacterium is gram-negative.

26. The preparation according to claim 25, wherein the
gram-negative bacterium is selected from a strain belong-
ing to the group consisting of the genera *Pseudomonas*,
10 *Hafnia*, *Hydrogenophaga*, and *Zymomonas*.

27. The preparation according to claim 26, wherein the
gram-negative bacterium is selected from a strain belong-
ing to the group consisting of the species *Pseudomonas*
15 *putida*, *Pseudomonas putida*, *Pseudomonas amyloclavata*,
Hafnia alvei, *Hydrogenophaga palleroni* (Basonym: *Pseudo-*
monas palleroni), *Moo5A10* and *Zymomonas mobilis*.

28. The preparation according to claim 24, wherein the
20 bacterium is gram-positive.

29. The preparation according to claim 28, wherein the
gram-positive bacterium is selected from a strain belong-
ing to the group consisting of the genera *Streptomyces*,
25 *Rothia*, *Bacillus*, *Kitasatoa* and *Bacteridium*.

30. The preparation according to claim 29, wherein the
gram-positive bacterium is selected from a strain belong-
ing to the group consisting of the species *Streptomyces*
30 *lydicus*, *Streptomyces nigrescens*, *Streptomyces sioyaen-*
sis, *Streptomyces platensis*, *Rothia dentocariosa*, *Bacil-*
lus badius, *Bacillus mycoides*, *Bacillus firmus*, *Bacillus*
aneurinolyticus, *Bacillus megaterium*, *Bacillus sp.*, *B.*
amyloliquefaciens, *Kitasatoa purpurea*, *Bacteridium sp.*
35 and *Bacillus megaterium*.

31. The preparation according to any of the claims 25-30,
wherein the transglutaminase exhibits optimum relative

activity at a pH of at least 6.5, preferably at least 7.0, more preferably at least 8.0, even more preferably at least 8.5, especially at least 9.0.

- 5 32. The preparation according to any of the claims 25-30, wherein the transglutaminase activity is inhibited by phenylmethanesulfonylfluoride (PMSF).

33. A parent transglutaminase derived from or producible
10 by *Streptomyces lydicus*, NRRL B-3446, or a functional analogue of said transglutaminase which

i) comprises an amino acid sequence being at least 80% homologous with the amino acid sequence of the parent
15 transglutaminase,

ii) reacts with an antibody raised against the parent transglutaminase, and/or

20 iii) is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding the parent transglutaminase.

34. The transglutaminase according to claim 33 which is-
25 derived from or producible by *Streptomyces platensis*, preferably DSM 40041, *Streptomyces nigrescens*, preferably ATCC 23941, or *Streptomyces sioyaensis*, preferably ATCC 13989.

30 35. The transglutaminase according to claim 33 or 34 which polymerizes α -casein.

36. A method for the production of transglutaminase comprising cultivation in a suitable nutrient medium a
35 strain belonging to any of the classes, orders, families, genera and species specified in any of the previous claims, especially *Streptomyces lydicus*, NRRL B-3446.

37. A transglutaminase composition comprising the transglutaminase preparation according to any of the claims 1-35 and a stabilizer.
- 5 38. A method of crosslinking proteins wherein a transglutaminase composition comprising the transglutaminase preparation according to any of the claims 1-35 is contacted with a proteinaceous substrate.
- 10 39. Use of the transglutaminase preparation according to any of the claims 1-35 in flour, meat products, fish products, cosmetics, cheese, milk products, gelled food products and shoe shine.
- 15 40. A DNA construct comprising a DNA sequence encoding an enzyme exhibiting transglutaminase activity, which DNA sequence comprises
- a) the DNA sequence shown in SEQ ID No. 1, and/or the DNA
20 sequence obtainable from the plasmid in *E. coli* DSM 10175, or
- b) an analogue of the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *E.*
25 *coli* DSM 10175, which
- i) is at least 80% homologous with the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *E. coli* DSM 10175, or
30
- ii) encodes a polypeptide which is at least 79% homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from the plasmid in *E. coli* DSM
35 10175, or
- iii) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified trans-

glutaminase encoded by the DNA sequence shown in SEQ ID No 1 and/or obtainable from the plasmid in *E. coli*, DSM 10175.

- 5 41. The DNA construct according to claim 40, in which the DNA sequence encoding an enzyme exhibiting transglutaminase activity is obtainable from a microorganism, preferably a bacterium.
- 10 42. The DNA construct according to claim 41, in which the DNA sequence is isolated from or produced on the basis of a DNA library of a strain of a gram-positive bacterium, preferably from an actinomycetes, more preferably from a strain of *Streptomyces*, or of a strain of a gram-negative
15 bacterium, preferably from a strain of *Bacillus* sp..
43. The DNA construct according to claim 42, in which the DNA sequence is isolated from or produced on the basis of a DNA library of a strain of *Streptomyces lydicus*, in
20 particular *Streptomyces lydicus*, NRRL B-3446.
44. A recombinant expression vector comprising a DNA construct according to any of claims 40-43.
- 25 45. A cell comprising a DNA construct according to any of claims 40-43 or a recombinant expression vector according to claim 44.
46. The cell according to claim 45, which is a microbial
30 cell, preferably a bacterial cell or a fungal cell.
47. The cell according to claim 46, in which the bacterial cell is a cell of a gram-positive bacterium, e.g. of the genus *Bacillus* or *Streptomyces* or a cell of a
35 gram-negative bacterium, e.g. of the genus *Escherichia*, and the fungal cell is a yeast cell, e.g. of the genus *Saccharomyces*, or a cell of a filamentous fungus, e.g. of the genus *Aspergillus*, *Trichoderma* or *Fusarium*.

48. The cell according to claim 47, wherein said *Escherichia* is *E. coli*.

49. The cell according to claim 47, wherein said *Aspergillus* is *Aspergillus niger*, *Aspergillus oryzae*, or *Aspergillus nidulans*.

50. The cell according to claim 47, wherein said *Bacillus* is *Bacillus licheniformis*, *Bacillus lentus*, or *Bacillus subtilis*.

51. The cell according to claim 47, wherein the cell belongs to a strain of *Streptomyces lydicus*.

52. A method of producing an enzyme exhibiting transglutaminase activity, the method comprising culturing a cell according to any of claims 45-51 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

20

53. An enzyme exhibiting transglutaminase activity, which enzyme

a) is encoded by a DNA construct according to any of claims 40-43,

25 b) produced by the method according to claim 52, and/or
c) is immunologically reactive with an antibody raised against a purified transglutaminase encoded by the DNA sequence shown in SEQ ID No 1 and/or obtainable from the plasmid in *E. coli*, DSM 10175, and being derived from
30 *Streptomyces lydicus*, NRRL B-3446.

1/3

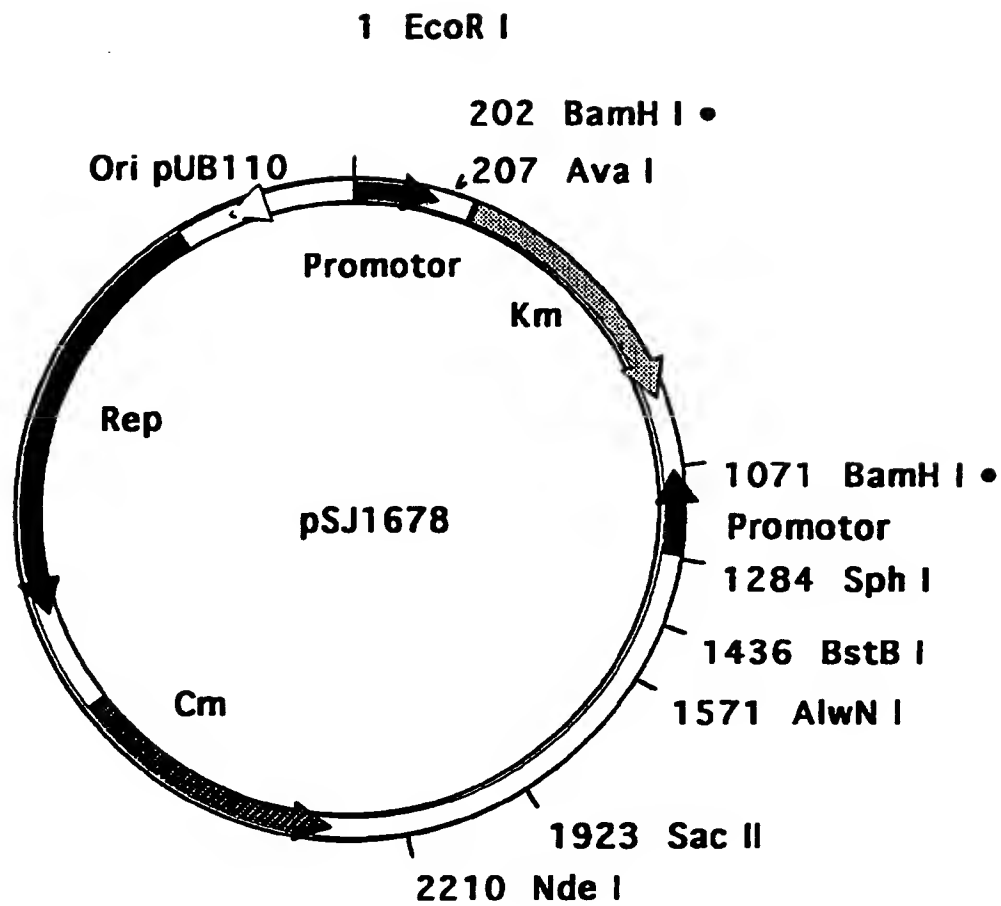


Fig. 1

2/3

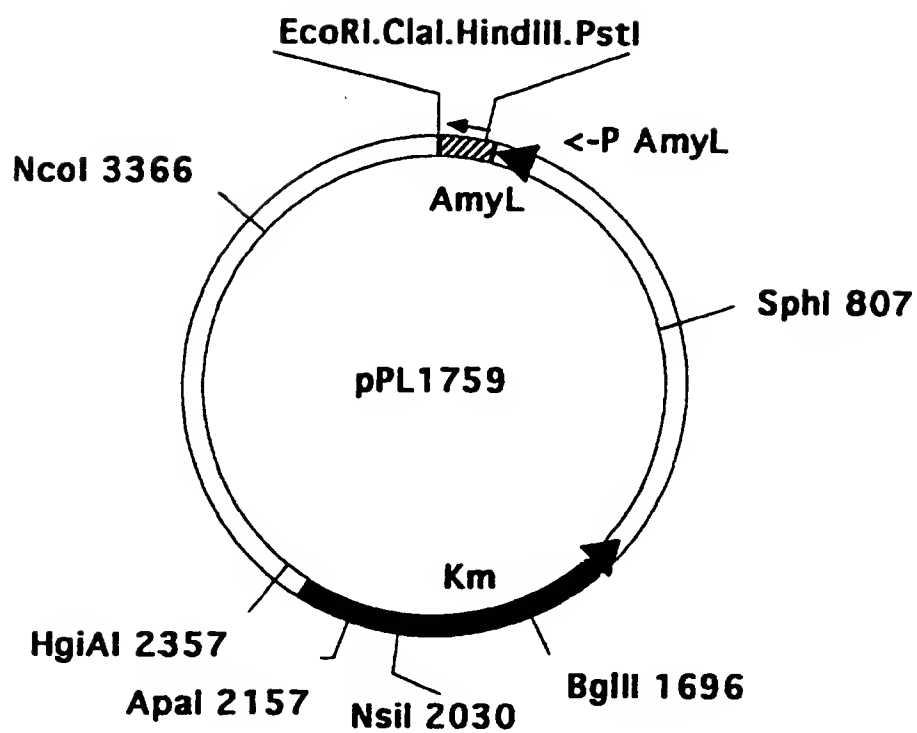


Fig. 2

3/3

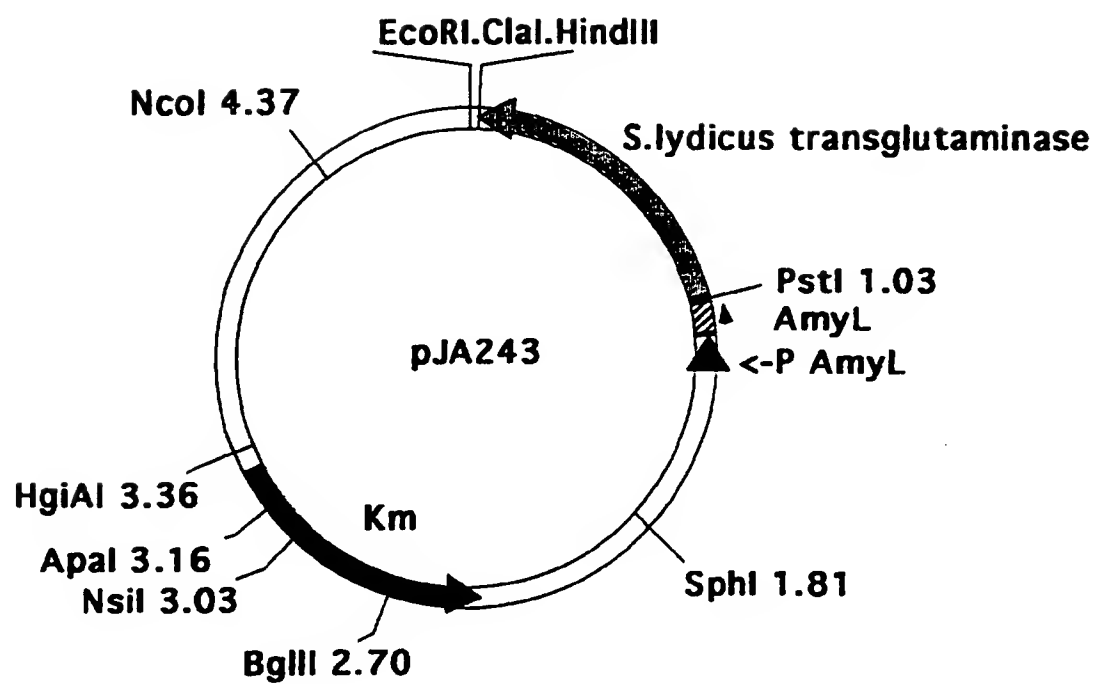


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00347

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/10, C12N 15/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, EMBL, GENESEQ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 116, No 23, 8 June 1992 (08.06.92), (Columbus, Ohio, USA), Klein, Janet et al, "Purification and partial characterization of transglutaminase from <i>Physarum polycephalum</i> ", page 331, THE ABSTRACT No 230570d, Bacteriol 1992, 174 (8), 2599-2605 ---	1-52
X	EP 0379606 A1 (AJINOMOTO CO.,INC.), 1 August 1990 (01.08.90) ---	1-52
X	Database Geneseq on Strand, Geneseq accession no. Q24197, AJINOMOTO: EP, A, 481504, COINC, 1992-10-09 ---	1-52

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

5 December 1995

Date of mailing of the international search report

12.12.95

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Yvonne Siösteen
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00347

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to "claim No.
X	Database Geneseq on Strand, Geneseq accession no. Q24201, AJINOMOTO: EP, A1, 481504, COINC, 1992-10-09 --	1-52
X	Chemical Abstracts, Volume 117, No 9, 31 August 1992 (31.08.92), (Columbus, Ohio, USA), page 642, THE ABSTRACT No 88752q, JP, 4108381, A, (Ando, Hiroyasu et al) 9 April 1992 (09.04.92) --	1-52
X	Chemical Abstracts, Volume 112, No 1, 1 January 1990 (01.01.90), (Columbus, Ohio, USA), page 618, THE ABSTRACT No 6095n, JP, 127471, A, (Motoki, Masao et al) 30 January 1989 (30.01.89) --	1-52
X	EP 0481504 A1 (AMANO PHARMACEUTICAL CO., LTD.), 22 April 1992 (22.04.92) --	1-52
A	US 5252469 A (HIROYASU ANDOU ET AL), 12 October 1993 (12.10.93) --	1-52
A	Chemical Abstracts, Volume 121, No 5, 1 August 1994 (01.08.94), (Columbus, Ohio, USA), page 733, THE ABSTRACT No 56249x, JP, 678783, A, (Anchi, Yoko et al) 22 March 1994 (22.03.94) --	1-52
A	Analytical Biochemistry, Volume 50, 1972, L. Lorand et al, "A Filter Paper Assay for Transamidating Enzymes Using Radioactive Amine Substates", page 623 - page 631; page 1 line 6 - line 9 -- -----	1-52

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/10/95

International application No.

PCT/DK 95/00347

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0379606	01/08/90	JP-A- 1027471 JP-C- 1817380 JP-A- 3043080 JP-B- 5023744 JP-A- 7075569 US-A- 5156956	30/01/89 18/01/94 25/02/91 05/04/93 20/03/95 20/10/92
JP-A- 4108381	09/04/92	US-A- 5252469	12/10/93
JP-A- 127471	30/01/89	NONE	
EP-A1- 0481504	22/04/92	JP-A- 5199883 US-A- 5420025	10/08/93 30/05/95
US-A- 5252469	12/10/93	JP-A- 4108381	09/04/92
JP-A- 678783	22/03/94	NONE	